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LII. SOME OBSERVATIONS ON THE HAEMOLYTIC ACTION OF CERTAIN QUININE SALTS ON THE ERYTHROCYTES OF DIFFERENT INDIVIDUALS AND ON THE RESISTANCE OF NEWLY FORMED RED CORPUSCLES TO HAEMOLYSIS UNDER THE INFLUENCE OF DISTILLED WATER.

By UPENDRA NATH BRAHMACHARI and PARIMAL SEN.

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By UPENDRA NATH BRAHMACHARI and PARIMAL SEN.

The following table is to be substituted for the one on p. 463.

Table showing the haemolytic action of the different salts of quinine upon the red corpuscles of different individuals.

	Quinine dihydro-chloride	Quinine hydro-chloride	Quinine disulphate	Quinine sulphate	Hydro-chloric acid	Sulphuric acid
Man	0·04	0·45	0·055	0·45	0·015	0·025
,,	0·05	0·5	0·07	0·5	0·02	0·03
,,	0·055	0·45	0·07	0·45	0·02	0·03
,,	0·06	0·5	0·0775	0·5	0·025	0·03
,,	0·05	0·37	0·07	0·4	0·015	0·025
,,	0·085	0·4	0·1	0·45	0·027	0·037
,,	0·08	0·3	0·095	0·325	0·03	0·035
,,	0·055	0·4	0·07	0·45	0·02	0·03
,,	0·085	0·4	0·1	0·5	0·035	0·045
,,	0·06	0·3	0·075	0·35	0·025	0·03
,,	0·045	0·45	0·06	0·5	0·02	0·03
,,	0·035	0·45	0·045	0·5	0·015	0·02
,,	0·06	0·3	0·075	0·35	0·025	0·03
,,	0·055	0·35	0·07	0·35	0·025	0·035
,,	0·07	0·3	0·08	0·35	0·03	0·035
,,	0·08	0·35	0·095	0·4	0·035	0·04
,,	0·055	0·3	0·07	0·35	0·025	0·03
,,	0·05	0·425	0·07	0·45	0·02	0·025
,,	0·065	0·35	0·075	0·35	0·03	0·03
Fowl	0·1	0·4	0·12	0·45	0·035	0·045

ERRATUM

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,,	0·055	0·45	0·07	0·45	0·02	0·03
,,	0·06	0·5	0·0775	0·5	0·025	0·03
,,	0·05	0·37	0·07	0·4	0·015	0·025
,,	0·085	0·4	0·1	0·45	0·027	0·037
,,	0·08	0·3	0·095	0·325	0·03	0·035
,,	0·055	0·4	0·07	0·45	0·02	0·03
,,	0·085	0·4	0·1	0·5	0·035	0·045
,,	0·06	0·3	0·075	0·35	0·025	0·03
,,	0·045	0·45	0·06	0·5	0·02	0·03
,,	0·035	0·45	0·045	0·5	0·015	0·02
,,	0·06	0·3	0·075	0·35	0·025	0·03
,,	0·055	0·35	0·07	0·35	0·025	0·035
,,	0·07	0·3	0·08	0·35	0·03	0·035
,,	0·08	0·35	0·095	0·4	0·035	0·04
,,	0·055	0·3	0·07	0·35	0·025	0·03
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By UPENDRA NATH BRAHMACHARI and PARIMAL SEN.

From the Campbell Medical School, Calcutta.

(*Received May 4th, 1921.*)

I. In the first part of this paper are recorded the observations made by us on the haemolysis of erythrocytes by certain salts of quinine in a series of patients taken promiscuously from the medical wards of a hospital of which one of us is in charge. The research was conducted with a view to determine whether the salts of quinine vary in their haemolytic action on the red corpuscles of different individuals and how far the quinine radicle or the acid portion of a quinine salt is responsible for the haemolysis.

Table showing the haemolytic action of the different salts of quinine upon the red corpuscles of different individuals.

	Quinine dihydrochloride	Quinine hydrochloride	Quinine disulphate	Quinine sulphate	Hydrochloric acid	Sulphuric acid
Man	0·040	0·45	0·055	0·45	0·015	0·025
"	0·050	0·05	0·007	0·50	0·002	0·003
"	0·055	0·45	0·007	0·45	0·002	0·003
"	0·006	0·05	0·0775	0·05	0·025	0·003
"	0·005	0·37	0·007	0·04	0·015	0·025
"	0·085	0·40	0·001	0·45	0·027	0·037
"	0·008	0·03	0·095	0·325	0·003	0·035
"	0·055	0·04	0·007	0·45	0·002	0·003
"	0·085	0·04	0·001	0·05	0·035	0·045
"	0·006	0·03	0·075	0·35	0·025	0·003
"	0·045	0·45	0·006	0·05	0·002	0·003
"	0·035	0·45	0·045	0·05	0·015	0·002
"	0·006	0·03	0·075	0·35	0·025	0·003
"	0·055	0·35	0·007	0·35	0·025	0·035
"	0·007	0·03	0·008	0·35	0·003	0·035
"	0·008	0·35	0·095	0·04	0·035	0·004
"	0·055	0·03	0·007	0·35	0·025	0·003
"	0·005	0·425	0·007	0·45	0·002	0·025
"	0·065	0·35	0·075	0·35	0·003	0·003
Fowl	0·001	0·04	0·012	0·45	0·035	0·045

We shall not enter here into the researches of previous workers on the haemolytic action of quinine salts. The reader is referred to them for comparison of our results on those points in which work has already been done by them.

In all our observations, the experiments were made with solutions of quinine salts in normal saline.

Each observation extended from $2\frac{1}{2}$ to 3 hours, the test tubes containing the mixture of washed corpuscles and quinine solution being kept at 40°. The corpuscles were washed with 0·85 % NaCl.

Columns (1), (2), (3) and (4) show the strength of the solution of a quinine salt (g. per 100 cc.) in which the red corpuscles begin to haemolyse in the course of $2\frac{1}{2}$ to 3 hours. Columns (5) and (6) show the percentage of HCl or H_2SO_4 in a solution calculated in terms of quinine dihydrochloride or quinine disulphate in which the same amount of acid is present. Thus 0·015 % hydrochloric acid means the amount of hydrochloric acid present in 100 cc. of normal saline containing 0·015 g. of quinine dihydrochloride.

From the above the following conclusions may be drawn:

(1) Quinine dihydrochloride is the most haemolytic of the quinine salts shown in the table.

(2) The acid salts are more haemolytic than the corresponding neutral salt.

(3) Free hydrochloric acid or free sulphuric acid is more haemolytic than a solution of quinine dihydrochloride or quinine disulphate containing the same amount of hydrochloric acid or sulphuric acid respectively.

It will be seen that the presence of the quinine radicle in a quinine salt retards the haemolytic action of the acid present in the salt.

From the above table it will be seen that the erythrocytes of different individuals vary differently in their resistance to haemolysis under the influence of a quinine salt and it is quite possible that we might come across an individual in whom the resisting power of the red corpuscles was still lower. Black-water fever is therefore likely to occur in those individuals in whom the erythrocytes are least resistant to the action of a quinine salt either owing to disease or to a natural weakness on their part in resisting the action of the acid portion of a quinine salt.

In our observations we have found that fowl's corpuscles are more resistant to haemolysis under the influence of a quinine salt than the average red corpuscles of man and therefore any conclusion drawn from observations on the haemolytic action of quinine salts in lower animals may be fallacious, if applied to the case of man.

Attempts have been made by us to determine whether the haemolytic power of a quinine salt is diminished by the presence of glucose in its solution. One such experiment has, up to the time of writing, been made in the case of the fowl and the following results obtained:

Commencing haemolysis of fowl's red corpuscles was obtained with:

(1) 0·1 % solution of quinine dihydrochloride, (2) 0·4 % solution of quinine hydrochloride, (3) 0·12 % solution of quinine disulphate all in normal saline.

(2) 0·12 % solution of quinine dihydrochloride, (2) 0·45 % solution of quinine disulphate, (3) 0·14 % solution of quinine disulphate, all in normal saline containing 5 % glucose.

It is thus seen that glucose retards the haemolytic action of salts of quinine.

II. The second part of this paper refers to a series of observations made to determine the resisting power of the red corpuscles present after repeated bleeding to haemolysis under the influence of distilled water.

In this Journal and in his *Studies in Haemolysis* one of us described a method of determining the fragility of the red corpuscles which may be briefly described as follows:

One part of blood is treated with two parts of distilled water and the proportion of haemoglobin in the undissolved corpuscles to that of the total volume of blood taken is estimated. This gives a factor which is fairly constant under normal conditions and may be termed the relative haemoglobin-value of the resistant corpuscles. In the case of the fowl, this factor was estimated to be 16·85 as an average of a large number of observations.

To obtain absolutely identical conditions, the blood of the fowl in each case was washed three times with 0·85 % NaCl solution, so that the osmotic pressure of the suspending fluid of the corpuscles in each case was exactly the same.

The corpuscles after repeated bleeding were obtained in the following way:—the fowls were repeatedly bled from their veins in the wings till the total quantity of blood removed amounted to 30 to 50 cc. The haemoglobin-value of the resistant corpuscles after repeated bleeding was estimated seven days after the last bleeding and compared with their haemoglobin-value before bleeding.

The following experiments were performed:

I. Weight of fowl—14$\frac{3}{4}$ oz. Total amount of blood removed after bleeding for four times = 27 cc. Haemoglobin-value of resistant corpuscles before bleeding = 17, after bleeding = 35·5.

II. Weight of fowl—3 lbs. 5$\frac{3}{4}$ oz. Total volume of blood removed after repeated bleeding for five times = 44 cc. Haemoglobin-value of the resistant corpuscles before bleeding = 15, after bleeding = 37.

III. Weight of fowl—3 lbs. 12 oz. Total volume of blood removed after repeated bleeding for four times = 34 cc. Haemoglobin-value of the resistant corpuscles before bleeding = 17, after bleeding = 32·5.

IV. Weight of fowl—3 lbs. 14 oz. Total volume of blood removed after repeated bleeding for six times = 54 cc. Haemoglobin-value of the resistant corpuscles before bleeding = 11, after bleeding = 20·5.

V. Weight of fowl—3 lbs. Total volume of blood removed after repeated bleeding = 41 cc. Haemoglobin-value of the resistant corpuscles before bleeding = 10, after bleeding = 17·7.

It will be seen that in all the above cases, the resistant corpuscles were increased after repeated bleeding, thus proving that red corpuscles obtained under these conditions are more resistant to haemolysis than under normal conditions.

LIII. A METHOD OF ESTIMATING PHENYLHYDRAZINE VOLUMETRICALLY AND ITS APPLICATION TO THE ESTIMATION OF PENTOSANS AND PENTOSES.

By ARTHUR ROBERT LING and DINSHAW RATTONJI NANJI.

(*Received May 13th, 1921.*)

The well-known method of estimating furfural by precipitating it as phenylhydrazone and weighing the latter, first suggested by G. de Chalmot and B. Tollens [1891], has the disadvantage that the phenylhydrazone is difficult to dry, principally owing to the fact that its melting point is below 100°. The alternative method of Kröber [1901] in which the furfural is precipitated and weighed as phloroglucide, leaves little or nothing to be desired on the score of accuracy, but it requires considerable time and attention, in addition to which phloroglucinol is an expensive substance. Flohil [1910] devised a method in which the reducing power of furfural towards Fehling's solution is made use of as a quantitative method. This method however even after the modifications suggested by Eynon and Lane [1912], and by Baker and Hulton [1916] is less accurate than the phloroglucide method.

Now since the sole difficulty of the phenylhydrazone method is that of drying the hydrazone, we sought to remove this difficulty by attempting to estimate the excess of phenylhydrazine, required to precipitate the hydrazone, volumetrically.

A method of estimating phenylhydrazine volumetrically has recently been suggested by Menaul and Dowell [1919]. These workers refer to the work of Ebler [1905], who found that the oxidation of hydrazine salts by cuprammonium salts proceeds in accordance with the equation

$$4\,(CuSO_4, 4NH_3) + N_2H_4, H_2SO_4 = 2\,(Cu_2SO_4, 4NH_3) + 2NH_3 + N_2 + 3\,(NH_4)_2\,SO_4.$$

Ebler suggests that on these principles a method of estimating copper or hydrazine may be based. Adan [1907] has shown that the reaction of phenylhydrazine with copper sulphate proceeds as follows:

$$C_6H_5.NH.NH_2 + 2CuSO_4 = H_2SO_4 + C_6H_6 + Cu_2SO_4 + N_2.$$

He found however that the reaction is accompanied by others in presence of Cl-ions owing to the formation of *p*-chlorophenylhydrazine and a diazo-compound.

Menaul and Dowell describe a gasometric method of estimating pentosans and pentoses. The method consists in distilling the substance with dilute

sulphuric acid, precipitating with phenylhydrazine acetate in presence of sodium sulphate (instead of sodium chloride) to diminish the solubility of the hydrazone, adding a 10 % solution of ammoniacal copper sulphate, and collecting and measuring the liberated nitrogen. This method has been adopted by the U.S. Department of Agriculture (see *Bull.* 49), it being there recommended however to precipitate the hydrazone in presence of sodium chloride.

It occurred to us that it might be more convenient instead of the gasometric method of estimating phenylhydrazine, to employ a titration method, and accordingly attempts were made to elaborate an iodometric method.

The reaction between iodine and phenylhydrazine was first studied by Emil Fischer [1877] who showed that it occurred in accordance with the following equation:

$$2C_6H_5.NH.NH_2 + 2I_2 = 3HI + C_6H_5NH_2.HI + C_6H_5N_3,$$

the products being hydriodic acid, aniline, and diazobenzene-imide. E. von Meyer [1887] showed that the reaction discovered by Fischer only occurs in presence of an excess of phenylhydrazine. When excess of iodine (at least four equivalents to one molecule of phenylhydrazine) is present, the reaction proceeds in accordance with the equation

$$C_6H_5.NH.NH_2 + 2I_2 = 3HI + C_6H_5I + N_2.$$

Our method for the estimation of pentoses or pentosans based on this reaction is carried out in the usual way so far as the distillation with acid is concerned.

In view of Menaul and Dowell's observation [1919], we carried out some experiments with sulphuric acid using a concentration of that acid equivalent to 12 % hydrochloric acid on the assumption that the ionisation of the sulphuric acid was 70 % of that of the hydrochloric acid. After eight hours' distillation however only 85 % of the possible furfural was obtained. It was therefore decided to use hydrochloric acid, and we found that the presence of sodium chloride and free acetic acid in no way affects the method.

After the accuracy of the method as compared with the phloroglucinol method had been established with solutions of furfural, it was applied to arabinose. A known weight of the pure sugar is distilled with 12 % hydrochloric acid in the usual manner until the distillate ceases to give the furfural reaction with aniline acetate paper. The distillation flask is heated in a sulphuric acid bath instead of by a direct flame, and the total distillate is made up to 250 cc. Of this 25 cc. is transferred to a 100 cc. flask, a drop of methyl orange solution added and the hydrochloric acid neutralised with 3*N* sodium hydroxide, care being taken during the neutralisation to avoid any rise of temperature. The solution is then acidified slightly with acetic acid, and 10 cc. of a standard aqueous solution of phenylhydrazine (approximately 2 %) added. The liquid is kept in a water bath at 50–55° for about twenty minutes when the precipitation of the hydrazone is complete. During

the precipitation the flask is closed with a cork, and the surface of the liquid reduced to a minimum by making it up with water to the neck of the flask. These precautions are taken in order to avoid oxidation of the phenylhydrazine by atmospheric oxygen. The solution is then cooled, its volume made up to 100 cc., and it is filtered through a hardened filter paper. The excess of phenylhydrazine is then determined in an aliquot portion of the filtrate. Into a flask is measured 10 cc. of $N/10$ iodine solution[1] and to it is added 10 cc. of the filtrate from the hydrazone. The liquid is then diluted with water to about 100 cc. and the excess of iodine is titrated with $N/20$ sodium thiosulphate, the volume in cc. being denoted by the symbol Y.

The titre of the original phenylhydrazine solution employed for the precipitation is ascertained in an exactly similar manner, and the number of cc. of $N/20$ sodium thiosulphate solution required is denoted by the symbol X. The number of cc. $N/20$ sodium thiosulphate equivalent to the furfural is $X - Y$.

The following are some results obtained with a sample of arabinose. It is seen that the results obtained by the new method agree closely with those obtained by the phloroglucinol method.

Weight of arabinose taken. g.	Weight of arabinose found by the phloroglucinol method. g.	Weight of arabinose found by the iodometric method. g.
0·250	0·248	0·250
0·250	0·249	0·250
0·232	—	0·2328

Some experiments were also carried out on the extract obtained by hydrolysing some Indian grasses with dilute sulphuric acid.

Extracts	Weight of pentoses found by the phloroglucinol method. g.	Weight of pentoses found by the iodometric method. g.
A	0·208	0·2095
B	0·152	0·1510
C	—	0·1860

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[1] The iodine solution we employed was 0·096 N.

LIV. ON CARRAGEEN (*CHONDRUS CRISPUS*). II[1].

ON THE OCCURRENCE OF ETHEREAL SULPHATES IN THE PLANT.

By PAUL HAAS.

Botany Department, University College, London.

(*Received May 10th, 1921.*)

Introductory.

Various views have from time to time been put forward concerning the composition of carrageen or Irish moss mucilage; the discordant results may be attributed to the fact that some of the investigators have worked on the weed as such, while others have examined an aqueous extract, apparently assuming either material to contain only a single substance. In all cases previous workers have contented themselves with establishing the presence of certain carbohydrate residues by hydrolysing the material or by distilling it with mineral acids or oxidising with nitric acid. Working on these lines Tollens and his pupils have shown that the substance contains galactose and fructose residues, the latter in such quantity as to induce Tollens [1914] to place carrageen among the fructosans, without however assigning any definite formula to the substance. Sebor [1900] on the other hand goes a little further in expressing the opinion that carrageen mucilage is a complex carbohydrate produced by the union of galactose, dextrose and levulose residues together with a small quantity of pentosan or methylpentosan as an impurity.

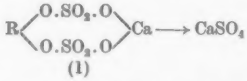
In the course of an investigation of the physical and chemical properties of an aqueous extract of Irish moss [Haas and Hill, 1921], it was however found that a hot water extract of the plant is not, as had been assumed by previous authors, a single substance but is composed of at least two constituents, which can be separated by making use of their different solubilities in cold water. A systematic investigation of these two substances, known for convenience as the cold and hot extracts, is now being undertaken, but although it is not as yet completed, the results so far obtained are thought to be of sufficient importance to justify publication at this stage.

It has long been known that *Chondrus crispus* yields on incineration a considerable amount of ash composed largely of calcium sulphate; practically all previous workers have drawn attention to this fact and have recorded

[1] For the first paper of this series see Haas and Hill [1921].

their inability to reduce the ash content by dialysis and, without offering any explanation for this anomaly, have confined their attention to the organic matter present, ignoring the possible significance of the high ash content.

Czapek [1920] commenting on the high percentage of sulphate found in the ash of many marine algae, states that it is not known what this is due to and a little later remarks: "Nicht unmöglich ist es, dass oft reichlich Gips vorhanden ist." That this remark does not apply in the present case is however now shown, by the fact that an aqueous extract of carrageen gives the reactions of the calcium ion but not of the sulphate ion; after hydrolysis, however, the addition of barium chloride produces a plentiful precipitate of barium sulphate showing that the calcium sulphate obtained by incineration is not present in the plant, as such, but is produced by the decomposition of an ethereal sulphate pre-existing in the undecomposed material. Owing to the bivalency of calcium such a compound must contain two sulphuric acid residues for each atom of calcium, as indicated by formula (1). The correctness of this assumption has been established by showing that the amount of sulphate in the solution after hydrolysis is double that found in the ash, since a compound of the type of formula (1) must on incineration lose half its sulphur while the remaining half is converted into calcium sulphate:



(1)

That this sulphur is really given off in the form of sulphuric acid on heating has been experimentally established and this decomposition accounts for the surprising readiness with which the extract of the weed tends to char when heated to constant weight in a steam oven.

While the occurrence of ethereal sulphates among products of animal origin is not uncommon, the same cannot be said of plant products and the present instance would appear to be the first recorded case of the existence of carbohydrate esters of sulphuric acid among plant products; apparently the only other known example of the occurrence of ethereal sulphates of vegetable origin is that of certain mustard oil glucosides which liberate potassium hydrogen sulphate on hydrolysis, but in any case the composition of these is very different from that of the substances here described.

Carrageen mucilage forms a very convenient material for demonstrating the ethereal sulphate grouping. since on boiling a little of the neutral solution with barium chloride no precipitate is formed; if a little concentrated hydrochloric acid is now added and the boiling continued a plentiful precipitate of barium sulphate is thrown down in the course of a minute or two.

The establishment of this ethereal sulphate grouping in carrageen necessitates a revision of many of the previously published statements concerning the composition of the water-soluble constituent of this material. Although it is not intended in the present instance to discuss the nature of the organic

complex with which the calcium sulphate residue is combined, it may be mentioned here that both the hot and cold extracts, as at present prepared, contain about 1 % of nitrogen, from which it has not as yet been found possible to free them.

Experimental.

At the outset of the present investigation an attempt was made to free the weed from adherent sea salt by a preliminary soaking in cold water; the first washings were distinctly saline but on removing these and allowing the weed to soak in distilled water all night it was found that the water had become quite syrupy and had extracted some material other than salts alone; the thick solution was therefore drawn off and replaced by fresh water and this process was repeated day after day until after about four weeks the amount of material extracted was practically negligible. The successive daily extracts were separately filtered and evaporated to dryness on tin lined copper pans and analysed.

The ash content of the thirtieth fraction was 21·5 % showing that this method could not be looked to for yielding an ash-free substance.

On digesting the residue from the cold water extraction with hot water over a boiling water-bath and evaporating the filtered solution the substance obtained was found to differ in some respects from that obtained from the cold water extraction, notably in the fact that strong hot water solutions gelatinise on cooling whereas the other material only produced thick gummy solutions, in equivalent strengths.

In view of the very tedious nature of the above described procedure and the fact that the hot extract was not entirely insoluble in cold water, the following improved method was adopted.

The handpicked weed was rapidly washed by squeezing it out a few times under a stream of running water and was then soaked for about an hour in distilled water. The aqueous extract was thereupon poured off and filtered, first through calico and then through a Chardin filter paper over a Buchner funnel. It was found advisable to replace the calico frequently owing to the clogging of the pores by the slimy particles of plant débris which were suspended in the liquid. The filtrate was evaporated to dryness over a water-bath; the residue so obtained is referred to for convenience as the Cold Extract or C.E.

The weed which remained after pouring off the water was then squeezed out as completely as possible and placed in a tall cylindrical vessel in which it was washed continuously for three days in running water by allowing the liquid to enter the vessel at the bottom and overflow at the top through muslin. At the end of this time the weed had lost its slimy feeling and was regarded as being practically free from any of the cold water extract; it was thereupon squeezed out, washed in several changes of distilled water and left to soak in the same over night; after one more squeezing out and washing it was spread out in thin layers and allowed to dry in the air. This exhaustively

extracted weed was then warmed with hot water over a water-bath and the resulting solution, after filtration and evaporation, yielded the so-called Hot Extract or H.E.

By collecting only the very first cold extract it was hoped to obtain a product as nearly as possible free from the H.E. since this dissolves but slowly in cold water, and by sacrificing most of the remaining C.E. together with some of the H.E. much time was saved and a fairly complete separation was effected. That the process is wasteful is shown by the fact that only about 37 % of the weight of the dry weed was obtained by this method, consisting of 14 % of C.E. and 23 % of H.E. respectively, whereas about 70 % of the weed is actually soluble in water.

Comparison between the Hot and Cold Extracts.

In external appearance there is no great difference between the two extracts; both form more or less transparent horny scales, although the H.E. is generally the clearer of the two; both are friable when dried in a steam oven but on exposure to moist air they absorb hygroscopic moisture and become soft and pliable; the amount of moisture taken up varies somewhat with the humidity of the atmosphere but the C.E. usually absorbs from 4 to 5 % more than the H.E.

When placed in cold water both swell up considerably, but the C.E. dissolves completely on stirring to form a gummy solution; the H.E., on the other hand, is only sparingly soluble in cold water and even if left to soak for a long time a large proportion of the material remains in the form of undissolved flakes; on warming, however, it dissolves fairly readily without however giving a perfectly clear solution; at a strength of about 1 %, such solutions, on cooling, show a tendency to gelatinise, although even a 2 % solution only forms a very thin jelly, and it is not until concentrations of from 3 to 5 % are reached, that a really stiff jelly is formed. The melting points of such jellies have been described elsewhere [Haas and Hill, 1921].

Towards salt solutions the behaviour of these two substances is different; thus while both the H.E. and the C.E. are precipitated from their solutions by half saturation with ammonium sulphate the H.E. only is precipitated by half saturation with sodium chloride or magnesium sulphate, the C.E. remaining unaffected thereby.

A further difference between the two is in their behaviour towards Rochelle salt; on adding five drops of a 20 % solution of this salt to 5 cc. of a 0·75 % solution of H.E. (which is too dilute to set to a jelly) the H.E. is thrown out in the form of a clear gelatinous precipitate which remains suspended in the aqueous medium, but on boiling and then setting aside to cool the whole liquid sets to a clear stiff jelly. Solutions of the C.E. are not affected in the same way. Other salts of the lyophilic series affect the H.E. to a less extent but observations on this subject are reserved for a future occasion.

Evidence of a chemical nature is also forthcoming to show that the C.E. and H.E. are different; this is based upon the colour reaction obtained from the two substances on warming them over a water-bath with alcoholic solutions of α-naphthol and sulphuric acid; the deep violet colour given by the H.E. points to the presence of a ketonic sugar which would appear to be absent in the case of the C.E.

The ash constituents of the Hot Extract.

Mention has already been made of the fact that all previous workers had commented on the high ash content of carrageen; the figures given by different authors vary from 14·15 % [Church, 1876], for the air dried material, to 20·6 % quoted by Czapek [1920].

In the present investigation the high ash content of the material was once more confirmed, as the following figures will show:

Ash in the untreated weed	14·6 %
„ „ cold extract	21·8 %
„ „ hot „	17·6 %
„ „ residue after extraction of all water-soluble material	5·19 %

These figures were in every case obtained by incineration of the oven-dried material. Some difficulty was experienced in these determinations owing to the tendency for the material to char quite suddenly while being heated in the steam oven to constant weight. That this should occur at the temperature of the steam oven was at first difficult to understand but was subsequently explained by showing that the material gives off sulphuric acid when it decomposes (see below, p. 475). This tendency to char adds considerably to the difficulty of accurate determinations of moisture content, more especially as the last traces of moisture are retained somewhat tenaciously.

Notwithstanding that earlier workers had recorded the fact that the ash content could not be reduced by dialysis and that one worker had actually observed an increase in the ash after dialysis [Moeller and Thoms, 1900], recourse was had to this method on several occasions. It was found that samples of the weed or of the C.E. which had a salt taste, lost it after dialysis with a corresponding falling off in the ash content; thus in one case the percentage of ash in a sample of C.E. was reduced from just under 40 to 21·79, but prolonged dialysis would not reduce it below the latter figure; the dialysing water was shown to contain appreciable quantities of chloride and sulphate and the experiment proved that while at least some of the ash constituents can be removed by dialysis, a considerable proportion of these resist such treatment. Similarly several days' dialysis did not reduce the percentage of ash in the H.E. below the average figure of 17·6.

With the object of establishing whether the reluctance to give up salts by dialysis was due to any inherent attraction of the colloidal material for inorganic salts in general, and sulphates in particular, samples of carrageen containing added sodium sulphate, carbonate or chloride were submitted to

dialysis and in every case these salts were completely removed after two or three days.

On the assumption that the reluctance to dialyse might be a peculiarity of the calcium sulphate a sample of H.E. was boiled with a solution of sodium carbonate, with the object of precipitating out the calcium, removing it by filtration and then dialysing away the sodium sulphate produced by double decomposition. On incinerating the dialysed material, however, it was found that the percentage of ash, instead of being reduced to zero, had been slightly raised, namely from 21·9 to 22·7[1]. The calcium had been successfully removed, since the ash now actually consisted of sodium sulphate, but, strange to say, this latter had not dialysed out. The slight increase, of course, is explained by the fact that the one atomic proportion of calcium (40) had been replaced by two of sodium (46).

An attempt was then made by reversing the order of procedure of the previous experiment to remove the sulphate radicle first, with barium chloride, and then dialyse away the resulting calcium chloride. On adding the barium chloride, however, no precipitate of barium sulphate was formed even after prolonged boiling, whereas on the addition of a little concentrated hydrochloric acid and boiling again for a few minutes, a heavy precipitate of barium sulphate was thrown down.

From these experiments it was concluded that the H.E. contains ionised calcium but that the sulphate complex is united to an organic residue and therefore only becomes ionised after hydrolysis.

That the calcium is, in fact, ionised was proved by showing that it can be quantitatively precipitated from solution by ammonium oxalate and that the estimations so carried out agree with those made on the ash obtained after incineration. The results obtained were as follows:

1. Ca by direct precipitation ... 5·47 %
2. „ incineration... ... 5·66 %

With regard to the sulphate residue it became necessary to assume the existence of a complex which would liberate ionised sulphate after hydrolysis, as indicated by the equation:

$$R\begin{matrix}O.SO_2.O\\O.SO_2.O\end{matrix}Ca + 2H_2O = R\begin{matrix}OH\\OH\end{matrix} + CaSO_4 + H_2SO_4$$

If this assumption is correct the amount of sulphate obtained by precipitation from the hydrolysed solution should be double that obtained by incineration, since the calcium present would only be sufficient to produce a single molecular proportion of calcium sulphate. That this assumption is borne out by experiment may be seen from the following figures:

	I	II
1. SO_4 by incineration...	12·15	11·8 %
2. „ hydrolysis ...	24·55	23·81 %

[1] The material used in this case was a mixture of C.E. and H.E., the experiment having been carried out before the improved method of separation had been devised.

The discrepancy between the figures in the two columns is due, no doubt to a difference in moisture content, arising from the difficulty experienced in drying the material without charring (see below). Notwithstanding this the ratio of the two figures in each column is pretty closely 1 : 2, as required by theory.

Although the hydrolysis by boiling hydrochloric acid is sufficiently rapid to enable one to observe it qualitatively in the course of a few minutes, by the addition of barium chloride, it was found that in order to ensure complete hydrolysis, it was necessary to boil for some hours. In the above analyses about 0·5 g. was dissolved in 100 cc. of water and boiled with 5 cc. of concentrated hydrochloric acid for at least five hours.

Attention has already been drawn to the fact that the H.E. tends to char when heated to constant weight in a steam oven. This can be accounted for on the above assumption concerning the form of combination of the sulphate complex; on decomposition, a compound of the type indicated by formula (1) should give off sulphuric acid, whereas calcium sulphate, if present as such in the plant, would not do so. On dry distilling some of the H.E. in a small tube and passing the evolved vapours into water a strongly acid solution was obtained which gave an immediate precipitate with barium chloride, showing that sulphuric acid was actually evolved.

Without for the present expressing any opinion upon the nature of the organic complex present in carrageen, the experiments above described justify the conclusion that the hitherto accepted views concerning the composition of this material require some modification; the water-soluble constituent of Irish moss should in fact not be regarded as a more or less complex carbohydrate contaminated with a certain amount of inorganic salts, but as a mixture of salts of sulphuric acid united to a carbohydrate yielding complex, in other words as a mixture of ethereal sulphates.

Summary.

1. The colloidal material extracted from *Chondrus crispus* by hot water consists of two substances.

2. These two substances may be separated from each other by taking advantage of their different solubilities in cold water.

3. The constituent which is more soluble in cold water produces thick viscous solutions; the other dissolves more readily in hot water and produces solutions which tend to gelatinise on cooling.

4. The Hot Extract is the calcium salt of an ethereal sulphate in which the calcium is freely ionised and can be quantitatively precipitated by the ordinary reagents for calcium, while the sulphate complex is not ionised until after hydrolysis of the compound. This accounts for the observation made by earlier workers that the high ash content of *Chondrus crispus* could not be reduced by dialysis.

It is hoped to continue the investigation of the constitution of both the hot and cold extracts and, if possible, also to extend the observations to other sea weeds.

In conclusion the author wishes to acknowledge his indebtedness to his friend and colleague Mr T. G. Hill for valuable criticism and advice during the course of this investigation and also to express his thanks to Miss Barbara Russell Wells for assistance with some of the analyses.

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LV. "FUMED" OAK AND NATURAL BROWN OAK.

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The well-known process for the production of a brown coloration on oak by exposure to fumes from a solution of ammonia, or to ammonia produced by the decay of nitrogenous organic materials, appears to have been practised for a very long time. Apart from the fact that oak darkened by the action of ammonia is usually regarded as having an appearance superior to that of stained oak, the chief advantages of "fuming" oak are that the effect extends some slight distance below the surface of the wood, the grain of the wood is not raised, and uniform colouring is obtained in depressions and angles of woodwork, which is often not the case where stains are employed.

It is to be noted that in addition to the production of large quantities of "fumed" oak artificially, it is well known to those interested in timber that part of the wood of certain oak trees in this country is of a deep reddish brown shade. Usually the colour of this natural brown oak is much redder than the brown oak produced by the action of ammonia, and the grey appearance of the latter is usually absent from this natural brown oak.

It is apparently impossible to tell whether or not a given tree will contain any of this natural brown oak until it is felled. Although many of these "brown" oak trees are found in the neighbourhood of London, few people are aware of their existence [A. L. Howard, 1920].

It has been shown by P. Groom [1915] that the coloration of natural brown oak is due to the growth of a fungus, though very little appears to have been done with regard to this coloration from a purely chemical standpoint.

In view of the wide application of the "fuming" process for darkening oak and other woods, and the possibility of some connection between "fumed" oak and natural brown oak, it seemed advisable to carry out some experiments on the subject. The results so far obtained in this investigation are dealt with below.

The action of ammonia on oak in the presence and absence of oxygen.

It is usually stated that the brown coloration obtained on oak subjected to the fumes from a solution of ammonia is due to the action of the ammonia on the tannin contained in the wood. In view, however, of the fact that alkaline solutions of tannin absorb oxygen, it appeared at the outset that the

coloration obtained in the "fuming" process was most probably due to the combined action of ammonia and oxygen from the air. That this view is correct may easily be demonstrated as follows: small pieces of oak are introduced into test-tubes filled with ammonia gas standing over mercury. The ammonia is rapidly absorbed by the wood, but in the case of most samples of oak practically no change in the appearance of the wood is noted. In some cases where English oak is employed, however, a reddish brown coloration is produced, the depth of colour depending on the amount of ammonia absorbed. This reddish coloration is referred to later.

If instead of pure ammonia a mixture of ammonia and oxygen is used, on introducing the oak the gas is absorbed less rapidly, and at the end of the experiment the pieces of oak showed the ordinary coloration of "fumed" oak.

On exposing the pieces of oak which have absorbed only ammonia to the air the characteristic brown coloration of "fumed" oak is also obtained. If, however, shavings of English oak be placed in a flask which is then evacuated, or the air removed by hydrogen or coal gas, on the introduction of ammonia gas a reddish coloration is obtained on the oak. If the flask is then sealed this coloration may be preserved. It would appear, therefore, that both ammonia and oxygen are necessary for the production of the brown coloration.

The usual practice adopted in "fuming" oak on a large scale is to subject the wood to the action of ammonia escaping from a concentrated solution of the gas, access of air being possible throughout the whole operation.

The absorption of oxygen by oak in presence of ammonia.

In order further to investigate the absorption of oxygen by oak, a Hempel "phosphorus" pipette was filled with thin oak shavings, which were then covered with a dilute solution of ammonia—0·88 ammonia solution diluted 1 in 5 with water. The results obtained for various samples of oak are given in the tables which follow:

Volume of oxygen absorbed by 10 *g. of oak shavings covered with a dilute solution of ammonia from* 100 *cc. of air in a given time.*

(*a*) English and Japanese oak:

Time in minutes	5	15	60	—	Overnight
English oak	2·6 cc.	5 cc.	10 cc.	—	21 cc. (*i.e.* complete absorption of oxygen)
Japanese oak	3	7	10	—	21 cc.

At the end of the experiment the oak shavings were removed from the pipette, washed with water and dried. In both cases the shavings had the appearance of "fumed" oak, though not of a very deep colour.

(*b*) Heart wood and adjoining sap wood from same piece of English oak; and sap wood of American oak:

Time in minutes	5	15	60	240	Overnight
Heart wood	3·4 cc.	6 cc.	10 cc.	17 cc.	21 cc.
Sap wood (English)	0	0·4	1·8	4·2	9·4
Sap wood (American)	0	0	0	—	2·0

In the case of sap wood only a very faint "fuming" effect was observed at the end of the experiment.

It is well known to those engaged in the process of "fuming" that sap wood is not darkened to any marked extent by the action of ammonia. This is due to the fact that tannin is present in such small quantity in the sap wood.

It can be shown that no substance is present in the sap wood that inhibits the action of the ammonia, by soaking the sap wood in a solution of tannin, drying and subjecting to ammonia and air, when the ordinary "fuming" effect is produced.

(c) White and brown English oak from the same tree and "fumed" Japanese oak shavings.

Time in minutes	5	10	15	60
White oak	2·9 cc.	4·8 cc.	6·4 cc.	12·6 cc.
Brown oak	1·6	2·0	2·6	5·5
"Fumed" oak	0·0	0·0	0·2	1·2

After the experiment the brown oak had lost its reddish appearance and was more like ordinary "fumed" oak.

It will be noted that the absorption of oxygen is small in the case of the brown oak and that practically no absorption of oxygen took place with the fumed oak.

In other experiments instead of ammonia solution a solution of caustic potash was used as the confining liquid. With a 3 % solution of potassium hydroxide the absorption of oxygen was more rapid than when ammonia solution was used, but at the end of the experiment the oak after washing and drying did not show any appearance of "fumed" oak, nor was the effect produced on subjecting the oak after this treatment to the action of ammonia. It should be noted in this connection that sometimes a solution of potassium hydroxide is used for purposes of staining oak.

The potassium hydroxide solution in the "phosphorus" pipette at the end of the experiment like the ammonia solution was strongly coloured, but unlike the latter became considerably lighter in colour on keeping for some days. On the addition of hydrochloric acid to the dark coloured ammonia solution a dark brown precipitate was obtained, but in the case of the potassium hydroxide solution the precipitate was only very slightly coloured.

The amount of ammonia absorbed by oak during the fuming process.

In order to determine the amount of this ammonia, thin shavings of different kinds of oak were exposed for several days to the action of excess of ammonia and oxygen. A current of air was then drawn over the "fumed" wood for several hours after the smell of ammonia was no longer apparent.

Determinations of nitrogen by the Kjeldahl method in the oak before and after "fuming," and of the ammonia evolved on distillation of the "fumed" oak with sodium hydroxide, gave results as follows:

	N in "unfumed" oak. %	N in "fumed" oak. %	Calculated % of NH_3 added	NH_3 obtained on distillation with NaOH. %
Japanese oak	0·353	1·75	1·69	1·64
English oak	0·308	2·716	2·9	2·23

It will be seen that very much more ammonia was absorbed by the English oak, which was very dark in colour, after "fuming," than by the Japanese oak, which had a lighter brown colour. In the case of the Japanese oak practically the whole of the ammonia absorbed was evolved on distillation with sodium hydroxide, but with the English oak towards the end of the distillation the evolution of ammonia was extremely slow, and after several hours the amount of ammonia obtained was considerably less than that absorbed. From the results obtained in these experiments, and on the assumption that ammonia solution of 0·88 sp. gr. contains 35 % by weight of ammonia gas, approximately 8 g. of this solution would be required for complete "fuming" of 100 g. of shavings of this sample of English oak. For a similar weight of wood in one piece a very much smaller amount of the solution is, of course, required in actual practice where the fuming effect is confined to the surface of the wood.

It was found that practically the whole of the ammonia absorbed by shavings of Japanese oak could be recovered by prolonged extraction of the wood with hot water, although after extraction for several weeks the shavings still had a slight brown colour.

The relation between the coloration obtained in the "fuming" process and the tannin content of various kinds of oak.

It is well known that some varieties of oak "fume" much more easily than others, and great variations are found in this respect in different samples of wood said to be of the same origin. Borings from six samples of oak were subjected at the same time to the action of ammonia escaping from a solution of the gas in a large bell jar. The samples of wood employed were English (A), English (B), Japanese, American, Sap English (B) and Sap American oak respectively. The intensity of the colour obtained decreased in the order given. In the case of the Sap American oak no appreciable effect was produced.

The tannin content of the various samples was compared by extracting 2 g. of the finely ground borings with 100 cc. of water, the solution being heated for some hours in the water bath. The tannin was determined by the Löwenthal method, and in the case of the first experiments no allowance was made for "non-tans" present. The results obtained were as follows:

"Tannin" in 100 cc. of extract:

English (A)	English (B)	American	Japanese	Sap English	Sap American
0·08	0·076	0·04	0·025	0·024	0·005

On comparing these results with those obtained for the "fuming" effect it will be seen that they are in the same order, but the difference between the "tannin" content of the American and Japanese oak is greater than was expected from the appearance after "fuming."

In a later experiment, however, in which allowance was made for "non-tans" in the solution, by a combination of the Löwenthal and hide powder

detannisation methods, the tannin content of the extract of the American oak was found to be almost identical with that of an extract of the Japanese oak.

Absorption of tannin from oak extracts by silk and subsequent "fuming" of the silk.

In view of the affinity of silk for tannin it appeared of interest to investigate the action of silk on the extracts obtained from the various kinds of oak dealt with above. Pieces of white "Jap" silk 3 by 3 inches were heated in the extracts for several hours, removed and dried. One piece from each solution was then suspended in a bell jar containing a concentrated solution of ammonia and left overnight.

In the case of the "unfumed" silk light colours were obtained, but after "fuming," the colours varied from dark brown in the case of English oak (A) extract, through lighter brown to colourless silk in the case of Sap American oak.

The colours obtained after "fuming" in the case of the pieces of silk treated with extracts of Japanese and sap English oak were of approximately the same intensity, as would be expected from the tannin content of the extracts. In the case of the extract of American oak the "fumed" silk was rather lighter in colour than was expected.

It was found that, as was expected, tannin is not taken up as readily from these extracts by cotton as by silk, but is absorbed by lustre-cellulose (viscose), a fine light brown coloration being obtained on drying and subjecting to ammonia. These colorations obtained in silk appear to be fast to light and stable to dilute alkalis, they are bleached very slightly by dilute mineral acids. Washing in water does not give a coloured extract, and in this respect the "fumed" silk differs from "fumed" oak which gives a highly coloured extract on treatment with water.

It should be noted that this strongly coloured aqueous extract from "fumed" oak does not dye silk, only a very faint coloration being obtained on silk treated with this extract, even after "fuming" of the silk so treated. In this respect "fumed" oak differs markedly from natural brown oak, the extract from which dyes silk very readily.

On treatment of silk, which has absorbed tannin from an extract of English oak with ammonia in absence of air, the red coloration obtained in the wood is not produced, but the ordinary "fuming" effect is noticed. It is found, however, that, as would be expected, oxygen is absorbed by silk in the process of "fuming" by means of a solution of ammonia.

The action of methylamine and trimethylamine on oak.

In view of the fact that there is some resemblance between the colour of natural brown oak and English oak which has been treated with ammonia in absence of air, it seemed advisable to investigate the action of alkyl am-

monias on oak; more especially as the decomposition products of the nitrogenous constituents of the wood are likely to contain substances of this nature.

English oak treated with methylamine in absence of air showed a fine reddish brown coloration, and if this gas mixed with a limited supply of oxygen is allowed to act on the wood the colorations of various samples of brown oak are exactly reproduced. It is found, however, that oak treated in this way gradually darkens in colour on exposure to air, until finally the ordinary coloration characteristic of "fumed" oak is obtained. The results obtained by exposing various kinds of oak to the action of methylamine and a plentiful supply of air are similar to those obtained with ammonia, with the exception that in the case of English oak the coloration obtained at the commencement of the experiment is redder than when ammonia is employed. Trimethylamine gave results similar to those obtained with methylamine. Silk which has absorbed tannin from an oak extract may also be "fumed" by these substituted ammonias.

In order further to investigate the production of the reddish coloration in oak treated with ammonia or a substituted ammonia in absence of air, pieces of English (C) and (D), Japanese, American and Austrian oak were placed in a glass tube. The air was then exhausted from the tube and methylamine from a solution of the substance allowed to enter. After some time a reddish coloration appeared on both pieces of English oak, a very slight similar coloration on the American oak, and still less on the Austrian oak, but none on the Japanese oak. It thus appears that English oak more readily assumes the red brown coloration of natural brown oak than do the other varieties. On admission of air after several weeks the red coloration rapidly changed to the brown characteristic of "fumed" oak.

I am indebted to Mr Hamilton Smith of Messrs Heal and Son, Ltd., for the samples of oak used in this experiment, and for other samples of wood to be used later.

Natural brown oak.

Various samples of natural brown oak were employed in this investigation, for some of which I am indebted to Prof. P. Groom, others were obtained from freshly felled trees near Rickmansworth.

It has been mentioned that the coloration of natural brown oak, which is different from that of ordinary "fumed" oak, can be reproduced by the action of ammonia or a substituted ammonia on English oak, if oxygen is partly or wholly excluded during the interaction. It has also been pointed out that the colour so far produced in this way darkens when the oak is exposed to air, which is of course not the case with natural brown oak. It is possible, however, that in the natural brown oak the tannin is combined with a base of greater complexity than the substances so far employed, and this red compound in the wood may not become oxidised on exposure to air. Various experiments were carried out in order to find out whether or not the

coloration of natural brown oak is due to the interaction of the tannin of the oak with such nitrogenous compound.

In the first place, in view of the fact that an organism is concerned in the production of natural brown oak it seemed possible this oak might contain more nitrogen than "white" oak, but so far no conclusive evidence has been obtained that this is the case, as will be seen from the results given below:

Percentage of nitrogen in various kinds of dry oak:

Brown oak (A)	(B)	English oak (A)	(B)	(C)	Japanese oak
0·404	0·343	0·388	0·344	0·331	0·386

These results are in all cases the mean of two determinations which were in close agreement. Although in some cases the amount of nitrogen in the "white" oak is as great as in the brown, it should be noted that the brown oak (A) and the white oak (C) are from the same tree, and in this case there is a considerable difference in the amount of nitrogen contained in the wood.

Examination of extracts from brown and white oak.

(*a*) *Aqueous extracts.* If the excess of nitrogen in the brown oak (A) as compared with the "white" oak (C) were due to the accumulation of ammonium compounds, it would be expected that considerably more ammonia would be obtained from an aqueous extract of brown oak than would be obtained from "white" oak extract on distillation with an alkali.

The amounts of free and albuminoid ammonia in 25 cc. of a cold water extract of shavings of the two kinds of oak were very small, but the ratio of the free ammonia in the extract of the brown oak to that in the other extract was 2·4 : 1 and the albuminoid ammonia 2·3 : 1. In very dilute hydrochloric acid extracts of the wood the ratios were similarly 2·1 : 1 and 1·4 : 1. Owing to the very small amounts of ammonia obtained in this way it is, however, impossible to draw any conclusion as to any connection between the ammonia content of these extracts and the colour of natural brown oak.

In view of the possibility that the coloration is due to the action of a nitrogenous substance other than ammonia, it was thought that evidence of such interaction might be obtained by determining the total nitrogen in extracts of brown and white oak, more especially as the nitrogen added to oak in the form of ammonia in the ordinary "fuming" process is removed when the "fumed" oak is extracted with water.

In this experiment 5 g. of cross grain shavings were shaken intermittently with 100 cc. of distilled water for three days. The nitrogen in the filtered extract was then determined by the Kjeldahl process.

Extracts from two samples of natural brown oak, English and Japanese oak, were employed in this experiment. The amount of nitrogen in the residues obtained from the extracts was very small in all cases, and although more nitrogen was found in the residue from the brown oak extract the amount of this nitrogen was not sufficient to justify any definite conclusion as to the part played by the nitrogenous constituents of the wood in the production

of the brown colour. Distillations in steam of "white" and brown oak from the same tree were carried out, but no appreciable differences were noted in the distillates so obtained.

It is to be noted that cold water extracts of brown oak contain only about two-thirds of the amount of acid (phenolphthalein as indicator) contained in similar extracts of "white" oak from the same tree. In view of the fact that absorption of oxygen by tannin with the production of coloured substances takes place in alkaline solution, the greater acidity of the "white" oak extract may be of some importance. This point will be investigated further from the point of view of hydrogen ion concentration of the extracts.

(*b*) *Alcoholic extracts.* 5 g. of cross grain shavings of two samples of brown oak, white English oak, and Japanese oak were extracted with 100 cc. of absolute alcohol for three days with intermittent mechanical shaking. As is the case with the aqueous extracts the alcoholic extracts from the brown oak are deeper in colour than the extracts from the white oak. This point is referred to later in connection with the ultra-violet absorption spectra of the extracts.

The nitrogen content in the residues obtained from the alcoholic extracts was extremely small in all cases, and was found to be rather less in the extracts from the brown oaks than the white.

It thus appears from these experiments that although a colour similar to that of natural brown oak can be obtained by the action of ammonia or a substituted ammonia on English oak under certain conditions, the production of this colour in nature is probably not entirely dependent on the nitrogenous constituents of the wood.

The dyeing of silk by means of extracts from natural brown oak.

It has been pointed out that an aqueous extract of natural brown oak, unlike the strongly coloured extract of "fumed" oak, dyes silk readily, but the silk dyed with an extract of natural brown oak already shows the colour of "fumed" silk, and is not altered in depth to any appreciable extent on exposure to ammonia and air. Further investigation of this point is in progress.

Effect on English oak of prolonged heating in the hot water oven.

It was found that if pieces of English oak were heated intermittently in a hot water oven for several weeks the colour of the wood gradually darkened until it became identical with that of some varieties of natural brown oak. It is known that coloured substances, "oak reds" or phlobaphenes, which occur in oak bark, and to a limited extent in oak, are produced by elimination of water from tannins. References to the production and properties of these substances are given by Freudenberg [1920]. Some of these tannin "anhydrides" are slightly soluble in water and others are insoluble. Their chemical constitution appears to be unknown. In order to investigate the possibility

of the colour of natural brown oak being due to the presence of "oak red," silk was treated with extracts of natural brown oak, English oak heated several weeks in the steam oven, and oak bark. Silk dyed in these extracts had, before "fuming," similar colours in the case of the natural brown and heated oak, but in the case of the bark extract the colour was much redder. The silk from the bark extract had, after "fuming," a colour quite different from ordinary "fumed" silk, and almost identical with that of the wood of the natural brown oak used in the experiment.

This result considered in relation to the results referred to in connection with the nitrogenous constituents of the oak, and the results obtained in the spectroscopic examination referred to later, would appear to indicate that the colour is partly due to the presence of oak reds, or phlobaphenes, and partly to the interaction of the tannin with a nitrogenous base in the wood, but further investigation is necessary before any definite conclusion can be reached.

The ultra-violet absorption spectra of oak extracts.

It was hoped that some information as to the cause of the coloration in natural brown oak might be obtained by a spectroscopic investigation of various oak extracts. For the use of the ultra-violet spectrograph employed I am indebted to the Government Grant Committee of the Royal Society. Photographs of the ultra-violet absorption spectra of extracts of "white" and brown English oak, heated English oak, and of oak bark were taken. In the solutions so far examined definite absorption bands were absent, but considerable general absorption was shown, more especially with brown oak and bark extracts. For investigation of the ultra-violet absorption spectra 1 g. of finely ground material was extracted with 100 cc. of water for 24 hours with intermittent heating. The extracts were filtered and diluted with an equal volume of water. The ultra-violet absorption spectra of the extracts of brown oak and oak bark from a "brown" oak tree were almost identical, and the absorption of the extract of heated English oak was intermediate between that of the extract of English oak and that of brown oak extract.

In the absence of characteristic absorption bands it is difficult to draw definite conclusions from these results, but they would appear to indicate that the natural brown oak and bark from a brown oak tree give up the same colouring matter to water, and that by heating the English oak some of this colouring matter is produced.

Summary.

The production of the brown coloration on "fumed" oak is due to the combined action of the ammonia employed and atmospheric oxygen. English oak treated with ammonia or a substituted ammonia in absence of oxygen shows a reddish coloration which is, however, changed on exposure to air with production of the ordinary coloration of "fumed" oak; other varieties

of oak either do not show this red coloration at all or only to a very limited extent.

The relation between the tannin content of various kinds of oak, and the coloration produced on "fuming," has also been investigated, together with the absorption of tannin from oak extracts by means of silk, and the subsequent "fuming" of the silk.

From the results obtained so far it appears that the reddish brown colour of natural brown oak may be due partly to the interaction of the tannin with a nitrogenous compound in the wood, and partly to the presence of a phlobaphene.

The investigation outlined above is being continued and extended to include the action of ammonia on other varieties of wood.

The author wishes to express his thanks to the Advisory Council of the Department of Scientific and Industrial Research for a grant for laboratory assistance, and to Miss P. Dumas for assistance in the analytical work.

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LVI. A METHOD FOR THE PREPARATION AND RECRYSTALLISATION OF OXYHAEMOGLOBIN.

By HAROLD WARD DUDLEY and CHARLES LOVATT EVANS.

From the National Institute for Medical Research, Hampstead.

(*Received June 6th, 1921.*)

Many methods for the preparation of oxyhaemoglobin in crystalline form have been described. In most of these the corpuscles, after having been separated from the serum, are laked by the addition of ether or of varying amounts of ether and alcohol, and from the solutions so obtained oxyhaemoglobin crystals are more or less readily deposited. These crystals, when blood yielding easily crystallisable oxyhaemoglobin is employed, are well formed and have the appearance of a pure substance. They are recrystallised by dissolving in water and adding alcohol or ammonium sulphate in appropriate quantities. But, although the crystals appear to be homogeneous, the analyses of oxyhaemoglobin prepared by these methods, even from the same species, vary very considerably, and in this connection it is interesting to note that, according to Abderhalden, oxyhaemoglobin which has been once recrystallised can still contain as much as 15 % of foreign protein.

One of us, wanting some pure oxyhaemoglobin, prepared a specimen from horses' blood by Abderhalden's method using alcohol as the reagent for bringing about crystallisation. Excellent crystals were easily obtained, but it was noted that it was never possible to bring the entire crystalline mass into solution in water. There was always an insoluble residue, indistinguishable from the soluble portion as far as crystalline character was concerned. After each recrystallisation a similar insoluble residue was encountered.

We suspected that this difficulty was due to the denaturating effect of alcohol on the protein, and we sought means to avoid completely the use of denaturating agents of every description, finally devising the following method, which up to the present we have used only in connection with horse corpuscles. It depends on the facts that corpuscles are laked by water and that oxyhaemoglobin is less soluble than haemoglobin in water.

Defibrinated horse blood is centrifuged and the corpuscles washed by aid of the centrifuge with isotonic saline solution until the supernatant liquid gives no turbidity when heated to boiling. The thick corpuscle paste is then transferred to collodion tubes and dialysed under the pressure of a column of mercury, first against running tap water for about three days, and finally against frequent changes of distilled water for about two days. The corpuscles

are completely laked and the haemoglobin becomes partly reduced during this operation, a deep purple solution being obtained. This is centrifuged and can then be poured off from the deposit of stromata etc. Oxygen is next bubbled through the clear solution until crystallisation of the oxyhaemoglobin occurs. As a rule this is somewhat delayed, crystallisation suddenly taking place after about 20 minutes' oxygenation. The pasty mass obtained is then centrifuged, the crystalline oxyhaemoglobin settling to the bottom of the tube as a thick scarlet paste. Under the microscope the crystals appear as very fine hair-like needles. The supernatant liquid is poured off. The crystalline oxyhaemoglobin so produced is *completely* soluble in water.

In order to recrystallise the material it is suspended in about two to three times its volume of water. The aqueous suspension is then warmed in a water bath to 37° and the containing flask is attached to a vacuum pump. Oxygen is pumped off and the oxyhaemoglobin reduced to haemoglobin, which, being much more soluble in water than oxyhaemoglobin, gives a clear, deep purple solution. This solution is then cooled and reoxygenated, when oxyhaemoglobin again crystallises out. The crystals are centrifuged down and the supernatant liquid discarded. This operation of recrystallisation can be repeated as often as may be desirable.

We propose to study the properties of oxyhaemoglobin so prepared and to apply the method to other bloods which give less easily crystallisable haemoglobins than that of the horse.

LVII. FEEDING EXPERIMENTS IN CONNECTION WITH VITAMINS A AND B:

(I) THE VALUE OF STEAM-DISTILLED PALM KERNEL OIL AS A CONTROL FAT.

(II) WHEAT BRAN AS A SOURCE OF VITAMINS A AND B.

By ARTHUR DIGHTON STAMMERS.

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(*Received June 9th, 1921.*)

(I) Some difficulty has been experienced in the past in obtaining a material, deficient in vitamin A, which can be relied upon to give a minimum of growth and thus furnish a control in feeding experiments upon other products.

It has been found that palm kernel oil, steam-distilled for a period of three or four hours at a temperature of 230° to 260° C., is entirely deprived of any vitamin A which it may originally have possessed. It is probable if not certain that any vegetable oil, if treated in this manner, would give the same results; the writer's experience, however, is confined to palm kernel oil.

Two curves are appended (Fig. 1) which show the growth obtained from this material. It will be observed from these that the total gain in weight recorded was in one case 22 g., and in the other 20 g. This, for all practical purposes, may be regarded as a minimum, and is borne out by other experiments now in progress in the writer's laboratory.

The experiments in each case extended over a period of three months, and 16 animals were involved, only four of which survived until the end of the experiment—these four showing marked signs of deficiency disease.

Donaldson's curves of normal growth are shown on the graph, and lettered *A* and *B* to correspond with the experiment to which they refer.

(II) The objects of the experiments to which this part of the paper relates were:

(*a*) to decide whether the administration of bran in the daily food of experimental animals would take the place of the antineuritic usually supplied; and

(*b*) to decide whether the sample used contained vitamin A.

The Medical Research Council's report makes it clear that bran is satisfactory as a source of vitamin B, but it is not so definite as regards vitamin A. On page 22, it is observed that an inconclusive result was obtained, while on page 102 a definite indication is given that bran is deficient in vitamin A.

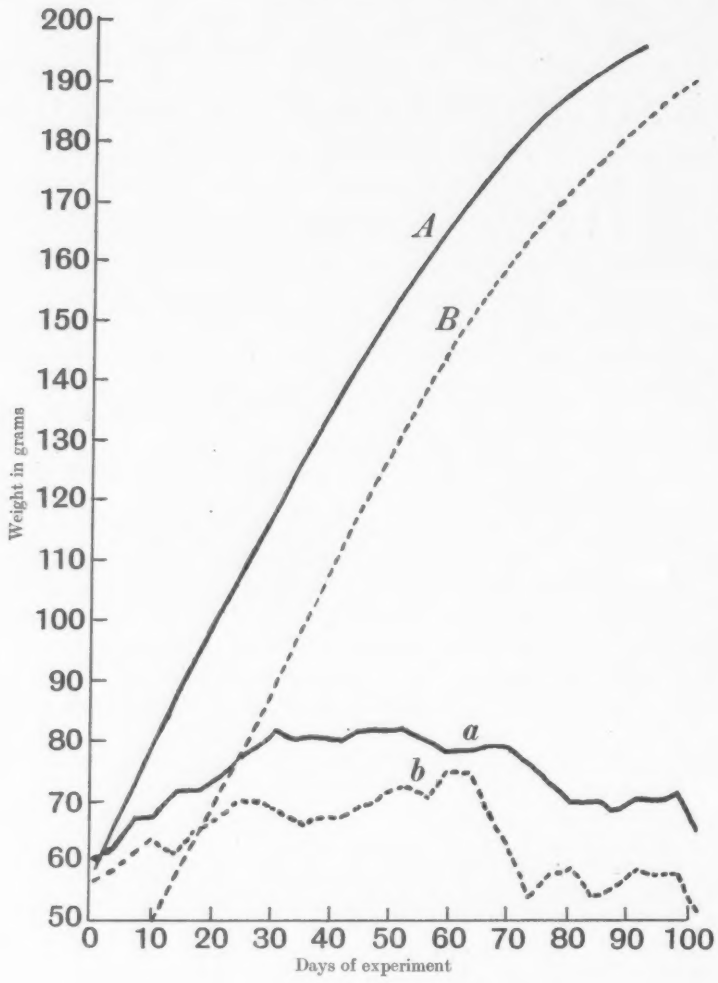

Fig. 1.

A, *B*. Donaldson's curves of normal growth.
a, *b*. Growth from steam-distilled palm kernel oil.

The experiments now to be described took place during the winter and the butter control which was used was therefore somewhat unsatisfactory; however, for purposes of comparison the results may be of interest, inasmuch as they bear out the hypothesis that bran does contain vitamin A and are mutually supporting in this respect.

Technique.

Twenty-four animals (rats) were used in all. Three sets of eight were fed upon:

1. Basal diet, steam-distilled palm kernel oil and wheat bran.
2. Basal diet, butter fat and wheat bran.
3. Basal diet and butter fat.

The bran used was an ordinary sample, obtained locally. In 1 and 2 no antineuritic was given, but 3 received the usual allowance.

The basal dietary used in this laboratory is as follows:

Purified caseinogen ...	26 %
Starch	54 %
Cane sugar	15 %
Salts	5 %

Ordinary commercial casein is used and treated by being spread out in thin layers in a hot-air chamber for some hours, subsequently being extracted with hot alcohol.

The salt mixture is based on McCollum's formula, with extensions suggested by Hopkins. It consists of:

NaCl	46·25
$MgSO_4$...	71·20
NaH_2PO_4	92·68
K_2HPO_4	254·60
$CaH_4(PO_4)_2.H_2O$ ...	144·20
Ca lactate	347·00
Ferric citrate ...	31·52
Sodium fluoride ...	·55
Manganese sulphate ...	2·00
Potassium iodide ...	10·00
	1000·00

Before feeding 85 parts of basal diet are taken and 15 parts of fat (appropriate to the experiment) are rubbed into the mixture. To this is added the antineuritic, which is prepared from marmite, in the following manner:

The crude marmite (250 g.) is shaken with 2 litres of 80 % alcohol for two hours on a shaker. The liquid is then decanted and filtered into a distilling flask, and the alcohol evaporated off under reduced pressure. The resulting fluid is filtered and made up to 250 cc. with distilled water.

Each animal receives 2 cc. of this per day. 1 cc. of lemon juice per day is also given, not that it is required for antiscorbutic purposes, but because the animals appear to grow better if this forms a constituent of the diet.

The animals receiving bran were given 2 g. each per day, and suitable allowance for the composition of this was made when preparing the food.

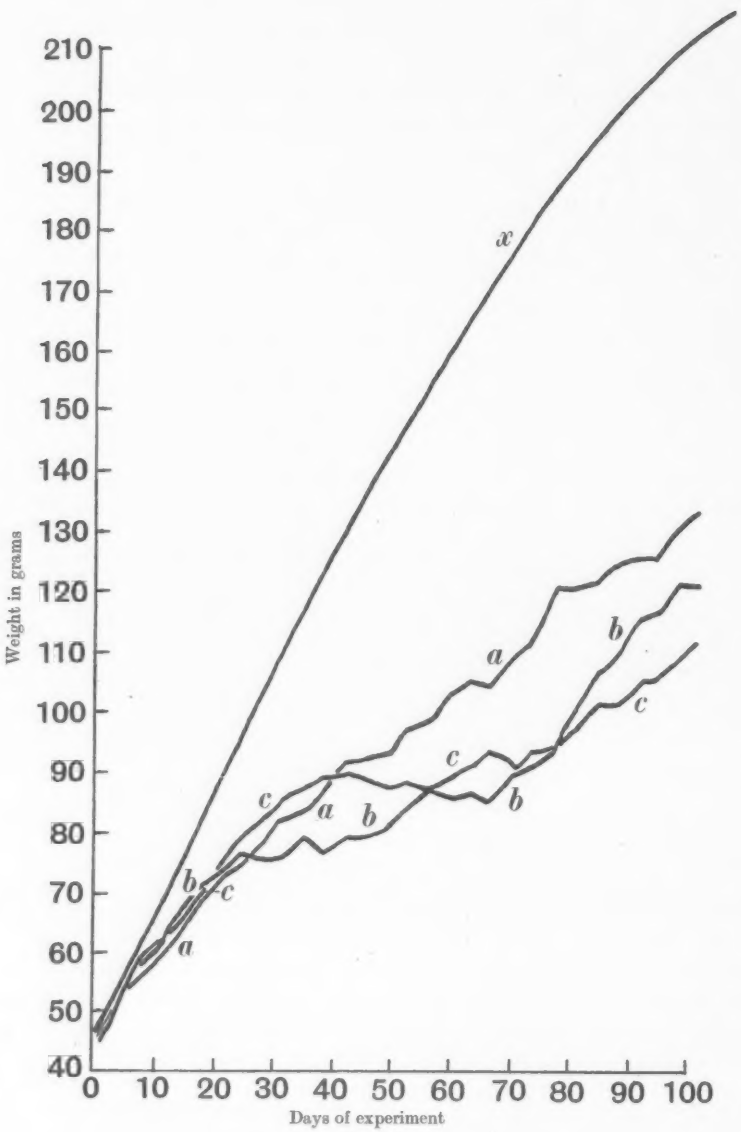

Fig. 2.

a. Bran + butter fat.
b. Butter fat.
c. Bran + palm kernel oil.
x. Donaldson's curve of normal growth.

Each animal receives, nominally, $1\frac{1}{2}$ g. of fat per day, with $8\frac{1}{2}$ g. of basal diet, but in practice it is given as much as it will eat, and it is endeavoured to give just such an amount as will cause a small quantity to be left. It is found that the actual amount eaten varies from 8 to 12 g. per day.

The animals were weighed twice a week, first thing in the morning before feeding and the experiment lasted for 101 days (from Oct. 19th 1920 to Jan. 28th 1921).

Results.

The growth curves are shown in the graph appended (Fig. 2). It will be seen that the average weight at the commencement was between 45 and 47 g. At the conclusion, the combination of bran and butter fat averaged 133 g., butter fat alone 120·5 g., and bran and palm kernel oil 112 g.

It will be seen, by reference to the curve of normal growth (Donaldson), which has been included in the graph, that the growth recorded was far below the normal. The heaviest animal at the conclusion of the experiment weighed only 150 g., whereas the normal for the age reached is 210 g.

None of the animals fed upon bran and palm kernel oil exhibited any traces of deficiency disease, and hence it appears certain that the sample of bran used contained sufficient vitamin A to give adequate resistance to disease if not to give normal growth. The palm kernel oil, being steam distilled, for four hours at 230° C., was beyond suspicion.

The curve denoting butter fat alone is a fair sample of those obtained in this laboratory during the winter and is probably, if not certainly, due to the butter being manufactured from cream obtained from stall-fed cows. Evidence is to hand that this was the case.

Throughout the experiment no trouble was experienced of such a kind as to indicate that the antineuritic properties of the bran were not adequate.

Summary.

Experiments made upon rats fed with mixtures of bran and butter fat and bran and palm kernel oil (steam-distilled) show that the sample of bran used undoubtedly contained vitamin A, the growth from the latter mixture being little inferior to that from butter fat.

The animals fed upon bran and palm kernel oil displayed no signs of deficiency disease although their growth was subnormal.

The butter fat control animals also showed a markedly subnormal growth and this is probably due to the cream from which the butter was made being obtained from stall-fed cows.

The bran and butter fat animals support the view that bran contains vitamin A by exhibiting a better growth than those fed on butter fat alone.

Throughout the experiment there were no symptoms which would give rise to the opinion that the antineuritic properties of the bran were inadequate.

LVIII. THE BEHAVIOUR OF PECTIN TOWARDS ALKALIS AND PECTASE.

By FRANK TUTIN.

From the University of Bristol Agricultural and Horticultural Research Station, Long Ashton, Bristol.

(*Received June 16th, 1921.*)

Pectin is a plant product which plays a part of considerable importance in the jam and fruit-preserving industries, and problems connected with it also arise in the making of wine, cider, perry and other fermented beverages made from fruit juices. It is present in fruits and also in roots, such as carrot, turnip etc., in which parenchymatous tissue is largely developed. A solution of pectin in presence of suitable quantities of sugar and an acid readily forms a gel, and it is to this property that the "setting" of jams and jellies is due. It may be prepared by the addition of alcohol to an aqueous extract of the respective fruit, when the pectin separates as a voluminous, gelatinous precipitate. Although the product so obtained cannot be considered in the strict, chemical sense as a pure compound no doubt can be entertained that it is, essentially, an individual substance. It cannot be crystallised, and the only methods of purification which have, so far, been found practicable are treatment in aqueous solution with animal charcoal and reprecipitation with alcohol.

The literature on the subject of pectin is voluminous, but a perusal of it reveals a great deal of confusion, and contradictory statements are frequent.

The present communication embodies the results of some preliminary work on pectin, and deals with the action of alkalis and of the enzyme, pectase, on this substance. It has long been known that pectin, on treatment with an alkali, yielded pectic acid, a substance which is insoluble in water in presence of an appreciable amount of an electrolyte. More recently Th. von Fellenberg [1914] has shown that the product obtained on distilling pectin with aqueous alkali contains methyl alcohol. Although he never made any thorough examination of the distillate he concluded, from indirect evidence obtained by several methods, that methyl alcohol was the only volatile product formed. To the present author, however, this conclusion did not seem to be justified, and investigation has proved this to be the case. Lippmann [1920] speaks of it as a well-known view that pectin is a methyl ester of "pectinic" acid, and attributes the presence of methyl alcohol in rum to the decomposition of pectin derived from the sugar cane.

No definite information appears, so far, to have been obtained regarding the action of the enzyme, pectase, on pectin beyond the fact that it causes a solution of the latter to set to a stiff gel, and that this change occurs more readily in the presence of chalk. The production of the gel has been considered to be due to the formation of calcium pectate.

The present author finds that the actions of cold, dilute alkali and of pectase on pectin are essentially identical. In both cases a salt of pectic acid is formed and not only methyl alcohol, but also acetone, is eliminated. In this connection it is worthy of record that both methyl alcohol and acetone have now been found to be present in cider, being doubtless derived from the pectin, which becomes destroyed during the fermentation of the apple juice.

Both the acetone and the methyl alcohol are eliminated with great facility from pectin, a small quantity of $N/10$ alkali at ordinary temperatures being sufficient to set free both compounds. This would suggest that both of them were present as ester groupings, the acetone occurring as an ester of its enolic modification, isopropenyl alcohol, $CH_3.C(OH) : CH_2$. It appears likely, therefore, that pectin is the dimethyl*iso*propenyl ester of pectic acid.

Experimental.

The pectin employed in this investigation, except where otherwise stated, was obtained from apples. Dried "pomace" (the "press cake" from the cider press) was heated to 100° with water and the liquid subsequently expressed. The pectin was then precipitated by the addition of alcohol to the dark-coloured solution, collected, pressed free from mother-liquor, and purified by solution in water and re-precipitation by alcohol. As thus obtained the pectin formed a brownish, voluminous, jelly-like precipitate, which could be deprived of a large volume of occluded mother-liquor by careful pressing in a cloth. Further purification was effected by treatment with animal charcoal in dilute aqueous solution. The first treatment with animal charcoal caused an appreciable diminution of the amount of material in solution, but subsequent treatments resulted in the absorption of only small quantities of the pectin, thus indicating that the initial loss was due essentially to the removal of impurities. On precipitating, the pectin after this purification separated as an almost colourless, jelly-like precipitate which, when dried, formed a greyish solid which could be reduced to a nearly white powder. The yield was nearly 5 % of the weight of the dried pomace employed.

The action of alkali on pectin.

All samples of pectin were found to possess a slightly acid reaction and the purified material (dried in a vacuum) when titrated, neutralised immediately in the cold 1·05 % of its weight of sodium hydroxide. On keeping, however, in aqueous solution at ordinary temperature in presence of an excess of the alkali a further amount of the latter equivalent to 12·35 % of the weight of

the pectin employed was taken up, the reaction being practically complete in half-an-hour, only a trace more alkali being neutralised on keeping for a further 18 hours. On then adding an excess of acid to the solution pectic acid was precipitated. When pectin was heated to about 80° with excess of $N/10$ alkali, however, much more of the latter was neutralised, considerable darkening occurred, and no definite end-point to the reaction could be ascertained. Moreover, on acidifying the alkaline mixture after heating for two hours no pectic acid was precipitated.

It has been recorded by von Fellenberg [1914] that pectin yields methyl alcohol when distilled with an excess of aqueous alkali, and this reaction was therefore investigated. A considerable quantity of pectin, in aqueous solution, was treated with an excess of sodium hydroxide and the mixture distilled in a current of steam for some time. It was at once evident that the distillate contained something other than methyl alcohol since it readily yielded iodoform on treatment with iodine and an alkali. The entire distillate[1] was therefore submitted to a systematic process of fractional distillation, employing a Young's rod and disc column, when it was separated into water and a fraction boiling below 70°. The latter, after drying over quick lime, was re-fractionated several times, again employing an efficient column, when it passed over from 56–66° and was finally collected in eight fractions, all of which were analysed. The fraction boiling at 66° consisted of methyl alcohol (found: C = 37·5, H = 12·6 %), whilst that boiling at 56–58° evidently consisted chiefly of acetone. It has a strong odour of the latter, gave C = 58·5, H = 10·8 %, and readily yielded acetonesemicarbazone, melting at 186°. The regular change in the composition of the intermediate fractions, as shown by analysis, clearly indicated that no third substance was present. The proportion of acetone to methyl alcohol produced appeared to be about one part of the former to two of the latter.

Pectin was then treated in cold, aqueous solution with 13·4 % of its weight of sodium hydroxide (the amount taken up by pectin in the cold), the mixture kept for 30 minutes, then made slightly acid, and distilled as before. It was then found that both acetone and methyl alcohol were formed, and to the same extent and in the same proportion as when the distillation had been conducted in the presence of a considerable excess of alkali. Moreover, when an amount of alkali sufficient to react with only half the pectin taken was employed both acetone and methyl alcohol were obtained, and apparently in the same relative proportions as when an excess of alkali had been used.

The action of pectase on pectin.

A quantity (13 g.) of pectin was dissolved in 2 litres of water, a few grams of precipitated calcium carbonate added, and then 40 cc. of pectase solution introduced. This was prepared by pounding fresh clover in a mortar with a

[1] The original distillate was always alkaline owing to the presence of a trace of ammonia, doubtless derived from a small amount of protein material present as an impurity in the pectin. It was neutralised before fractionation.

little water and then expressing the liquid from the resulting mass. On keeping over night the whole mixture set to a jelly. The latter was broken by vigorous shaking and distilled in a current of steam. On fractionating the distillate it was found to contain acetone and methyl alcohol to the same extent as if the pectin had been distilled with alkali.

The contents of the distillation flask were then filtered, and the filtrate concentrated to a small bulk under diminished pressure and precipitated with alcohol. A small quantity of an amorphous solid was thus obtained, which was not unchanged pectin, but probably represented impurities in the original sample of pectin used, whilst the filtrate from this amorphous solid contained only a very small amount of material, largely dextrose, which was doubtless derived from the clover extract which had been added. Pectin, when treated with pectase in the presence of chalk[1], therefore appears to yield only acetone, methyl alcohol and the gelatinous solid which consisted of calcium pectate, as shown below. The action of the enzyme, pectase, on pectin is therefore precisely analogous to that of sodium hydroxide, since the latter has been shown to convert pectin into acetone, methyl alcohol, and sodium pectate.

The solid particles of jelly which had been separated by filtration from the liquid in the distillation flask were macerated in the cold with dilute hydrochloric acid, the mixture filtered and the solid washed until free from calcium. After treatment in solution as sodium salt with animal charcoal the acid was reprecipitated, washed, dried in a vacuum and analysed. Found: C = 39·2, H = 5·1, NaOH neutralised = 20·86 %. Pectic acid prepared by the action of sodium hydroxide in the cold on pectin gave C = 39·4, H = 5·1, NaOH neutralised = 20·90 %.

Pectin from other sources.

In order that the pectin from plants belonging to different Natural Orders might be compared with the product from apples, carrots, turnips and mangold wurzels were investigated. It was then found that the pectin from each of these sources behaved like that from apples when treated with pectase or with alkali, yielding in each case both acetone and methyl alcohol.

The product obtained from mangold wurzels, however, was obviously much less pure than apple pectin, and yielded quite an appreciable amount of ammonia when distilled with alkali.

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[1] Chalk was added since the presence of free acid retards the action of pectase.

LIX. FURTHER OBSERVATIONS ON THE DIGESTION OF FIBRIN AND CASEINOGEN BY TRYPSIN.

By EDWARD STAFFORD EDIE.

From the Physiology Department, Aberdeen University.

(*Received June 18th, 1921.*)

In a previous paper [Edie, 1919] it was shown that the activity of trypsin as measured by its digestive action on fibrin and on caseinogen is affected to such a different degree by alcohol as to make it seem either that two enzymes are concerned, or, if only one enzyme, that the two substrates are acted on by different groups or side chains, one group being much more sensitive than the other. The effect of heat on the digestion of fibrin and caseinogen by trypsin was next studied. It had been found previously [Edie, 1914] that trypsin when boiled in acid solution still retains much or in some cases all of its power of hydrolysing caseinogen, but in only one case was its action on fibrin tested after heating in this way. Digestion of the fibrin was still noticed, but the amount even in the case of the unheated trypsin was so small that a fresh series of experiments was undertaken.

The trypsin solutions were generally prepared by extracting finely minced sheep's pancreas with water and a little chloroform for 10 to 14 days and filtering. A little chloroform was then added as a preservative. The experiments were carried out as previously detailed.

In the experiments described in the previous paper it had been found that in presence of $N/25$ to $N/50$ HCl the trypsin solutions used retained 60 to 100 % of their original digestive power, as tested on caseinogen, after being heated to 100° for three minutes. The same treatment was applied to the trypsin solutions in this series of experiments and the results are shown in the table.

In making these extracts, one part of pancreas was extracted with two parts of water in each case.

In each experiment 1 cc. trypsin + 40 cc. 0·5 % Na_2CO_3 + 1 g. fibrin were used on the one hand, digestion being for three hours, and 1 cc. trypsin + 40 cc. 1·5 % caseinogen in 0·5 % Na_2CO_3 on the other hand, digestion being for one hour.

The amount of digestion was estimated by precipitating unchanged caseinogen with tannic acid, or by filtering off the undissolved fibrin in the different sets of experiments respectively, and determining the nitrogen in

the filtrate by Kjeldahl's method. Control experiments were always carried out at the same time.

No.	Reaction (HCl)	Treatment	Fibrin digested (in cc. of $N/10$ nitrogen)	Caseinogen digested
1	$N/55$	3 min. at 100°	0·0	1·2
		Control	20·1	28·6
2	$N/20$	1·5 min. at 60°	0·0	3·1
		Control	21·9	34·7
3	$N/20$	1·5 min. at 60°	0·0	5·1
		Control	22·1	30·9
4	$N/30$	1 min. at 85°	0·0	1·9
		Control	7·1	22·4
5	$N/30$	1 min. at 65°	0·0	3·2
		Control	8·6	22·9
6	$N/20$	0·75 min. at 100°	0·0	3·2
		Control	21·2	34·6
7	$N/20$	0·75 min. at 66°	2·2	19·5
		Control	16·6	34·3
8	$N/20$	1·5 min. at 66°	1·1	8·3
		Control	16·2	31·8
9	$N/40$	1·5 min. at 75°	3·4	17·0
		Control	17·4	37·4

In the above experiments it will be seen that even in acid solution the pancreatic extracts used generally lost practically all their power to digest fibrin even when heated only to 60° for a minute and a half. The power to digest caseinogen was not so completely lost, especially in the last three experiments, but it was so markedly reduced, compared with what had been previously found, that the matter merited further investigation.

The extracts used above were aqueous, so a few experiments were carried out to compare the effect of heat on aqueous and alcoholic (20 %) extracts of the same pancreas. The experiments were carried out in the same way as those previously noted.

No.	Reaction (HCl)	Extract	Treatment	Fibrin digested cc.	Caseinogen digested cc.
10	$N/40$	Aqueous	1·5 min. at 75°	3·4	17·0
		,,	Control	17·4	37·4
		Alcohol	1·5 min. at 75°	17·3	29·3
		,,	Control	31·0	47·2
11	$N/50$	Alcohol	1 min. at 100°	23·4	34·4
		,,	Control	27·9	42·8
12	$N/40$	Aqueous	1 min. at 100°	0·0	—
		,,	Control	11·8	—
		Alcohol	1 min. at 100°	21·1	—
		,,	Control	23·1	—
13	$N/40$	Aqueous	1 min. at 100°	0·4	—
		,,	Control	11·7	—
		Alcohol	1 min. at 100°	14·0	—
		,,	Control	21·5	—

It will be seen that the alcoholic extracts retain very much more of their power to digest fibrin after being heated than do the corresponding aqueous extracts. The amount of protein and other nitrogenous substances was practically the same in the two sets of extracts. It might be supposed that the alcohol itself in some way protected the trypsin from destruction when

heated, the lower boiling point of alcohol preventing the alcoholic extract from reaching such a high temperature in the water-bath as the corresponding aqueous extract would reach. No such protection was found to be afforded by alcohol, however. Corresponding aqueous and alcoholic extracts were diluted with alcohol and water respectively so that the percentage of alcohol was the same in both. They were then heated to the same extent and tested on fibrin.

No.	Reaction (HCl)	Extract	Treatment	Fibrin digested cc.
14	*N*/40	Alcoholic	1 min. at 100°	21·1
		,,	Control	23·1
		Aqueous, alcohol added afterwards	1 min. at 100°	0·0
		,,	Control	18·9
15	*N*/40	Alcoholic	1 min. at 100°	14·0
		,,	Control	21·5
		Aqueous, alcohol added afterwards	1 min. at 100°	0·0
		,,	Control	7·9

These experiments show that the addition of alcohol to an aqueous extract of pancreas does not afford any protection against heat to the trypsin as measured by its action on fibrin.

In the original experiments on the resistance of trypsin solutions to heat, the solutions, when heated, contained only a very small amount of nitrogen, not more, in some cases, than 0·02 %. The pancreatic extracts used in the experiments now described were very much richer in nitrogen and generally contained 15 to 20 times as much as the older extracts. This corresponds to a considerable amount of protein in the solution and as protein is known to form a loose compound with hydrochloric acid it was decided to try the effect of considerably higher amounts of acid in the solutions to be heated.

It was now found that the protection afforded to trypsin solutions when heated depends on the amount of acid present, and the more protein there is in solution, the more hydrochloric acid must be added to prevent the trypsin being destroyed by heat.

The following experiments show this increasing protection with increase of acid:

No.	Reaction (HCl)	Treatment	Fibrin digested cc.	Caseinogen digested cc.
16	*N*/50	1 min. at 100°	5·6	21·1
		Control	16·9	39·1
17	*N*/23	1 min. at 100°	—	25·1
		Control	—	35·7
18	*N*/17	1·5 min. at 100°	7·4	26·8
		Control	7·6	26·7
19	*N*/15	3 min. at 100°	10·9	26·2
		Control	10·9	26·4
20	*N*/15	2 min. at 100°	3·5	19·2
		Control	11·6	28·7
21	*N*/12	2 min. at 100°	8·4	14·8
		Control	12·2	15·6

Experiments 16 to 19 show the effect of using increasing amounts of hydrochloric acid with the same trypsin preparation, and experiments 20 and 21 show this again with another sample of trypsin.

Similar results have been obtained with many aqueous extracts of pancreas prepared in the laboratory, and it may be stated generally that the higher the proportion of nitrogen contained in such an extract the larger the amount of hydrochloric acid which must be added in order to prevent destruction of the trypsin by heat.

In a few cases, as for example experiments 18 and 19, it was found that the protection against heat afforded by a certain amount of acid was the same as regards digestion both of fibrin and of caseinogen. Usually, however, the destruction of the fibrin digesting power was considerably greater than that of the caseinogen digesting power.

It has been pointed out by Mellanby and Woolley [1913] that in acid of the strength of 0·05 *N* (HCl) trypsin is slowly destroyed at 16° and more rapidly at 35°. Apparently at room temperature about half the trypsin is destroyed in four hours, and two-thirds is destroyed in a day. The activity of the trypsin in their experiments was measured by its power of coagulating calcified milk. Other references to the effect of hydrochloric acid on trypsin at moderate temperatures have been mentioned in a previous paper [Edie, 1914]. It was also found by Lénard [1914] that if trypsin is rendered inactive by addition of acid, only a trace of its activity is restored by neutralising and then adding alkali. These observers appear only to have tested the activity of the trypsin on one substrate, but in the following experiments the action of hydrochloric acid on trypsin at room or body temperature has been tested as regards the power to digest both fibrin and caseinogen. In these experiments alcoholic (15 %) extracts of pig's pancreas were used. These were practically neutral. In every case 1 cc. of the original trypsin was compared with that quantity of the trypsin + acid which would contain 1 cc. of trypsin originally, and the solutions so adjusted as to contain the same amount of sodium chloride. Digestion both of fibrin and of caseinogen was carried on in presence of 0·5 % sodium carbonate, 1 g. fibrin or 0·6 g. caseinogen being used, in about 40 cc. of fluid. The amount of digestion is expressed, as usual, in cc. of *N*/10 nitrogen.

22. 10 cc. trypsin + 20 cc. *N* HCl. Kept at 36° for 12 min. 20 cc. *N* NaOH then added.
Digestion by control 21·6 cc. fibrin, 38·2 cc. caseinogen.
„ treated trypsin 0·0 cc. „ 8·8 cc. „

23. 20 cc. trypsin + 10 cc. *N* HCl. Room temperature for 4 days. 10 cc. *N* NaOH added.
Digestion by control 12·1 cc. fibrin, 31·6 cc. caseinogen.
„ treated trypsin 0·0 cc. „ 5·4 cc. „

24. 40 cc. trypsin + 20 cc. *N* HCl. Room temperature for 11 days. 20 cc. *N* NaOH added.
Digestion by control 11·7 cc. fibrin, 38·7 cc. caseinogen.
„ treated trypsin 0·0 cc. „ 5·6 cc. „

It will be seen from these experiments that the power of trypsin to digest fibrin is destroyed considerably more readily in acid solution at moderate

temperatures than is the power to digest caseinogen. It is also seen that the power to digest caseinogen withstands a considerably higher percentage of hydrochloric acid than has generally been supposed, the strength of acid being $N/3$ in these experiments and several others with similar results. In one experiment, after 24 hours at room temperature in $N/3$ hydrochloric acid, the trypsin still retained about 10 % of its original fibrin digesting power, but otherwise no fibrin was digested at all after treatment of the trypsin with acid of this strength for a day or upwards.

On the whole, then, treatment of trypsin solutions with hydrochloric acid either at high or low temperatures shows that the power to digest fibrin is more readily destroyed than the power to digest caseinogen. This bears out the theory discussed previously [Edie, 1919] that in some respects the fibrin digesting power is the more subject to outside influences and again points to the hydrolysis of fibrin and of caseinogen being carried out by different side chains, those digesting caseinogen being the more stable.

In my previous paper [1919], the work of Fermi was referred to as showing that after treatment with various reagents trypsin would no longer digest fibrin but would still digest gelatin. Pollak was also mentioned as finding that with different enzyme preparations the relative amounts of serum and gelatin digested varied enormously. I have also found that the relative amounts of fibrin and caseinogen digested by different trypsin solutions vary very much, and this without subjecting the enzyme to treatment of any kind.

Thus, three enzyme solutions were prepared in exactly the same way, by extracting minced sheep's pancreas with three times its weight of water for 14 days and filtering. These were compared at the same time. 1 cc. of each trypsin was taken, with 40 cc. 0·5 % Na_2CO_3 and 1 g. fibrin on the one hand, and 1 cc. trypsin with 40 cc. 1·5 % caseinogen in 0·5 % Na_2CO_3 on the other hand. The amounts of digestion in the three cases were as follows:

Enzyme	Fibrin digested in 2 hours. cc.	Caseinogen digested in 1 hour. cc.
1	12·4	33·7
2	16·1	32·9
3	3·3	17·6

The differences in the relative amounts of fibrin and caseinogen digested by these enzymes, especially Nos. 2 and 3, are very marked. Similar results were frequently noticed in other cases, the general rule being that considerable amounts of caseinogen were digested even though a particular enzyme solution had little or almost no action on fibrin.

When enzyme solutions were kept for some time it was found that the fibrin digesting power as a rule diminished to a very much greater extent than the caseinogen digesting power. For example, a freshly prepared trypsin, under the usual conditions, digested 16·1 cc. fibrin and 32·9 cc. caseinogen. In 15 months, under the same conditions, this trypsin digested 4·8 cc. fibrin and 18·5 cc. caseinogen.

In one extreme case I examined a solution of trypsin which had been in the laboratory for over ten years. It had no digestive action on fibrin at all, but still digested caseinogen to the extent of 26·6 cc. under the usual conditions.

These facts afford further evidence that the digestion of fibrin and of caseinogen by pancreatic extracts is either due to different enzymes or at least to different side chains if only one enzyme is involved. In my previous paper [1914] I mentioned that the power to digest caseinogen seemed to be less affected by heat than the power to coagulate milk, which was taken as the measure of activity of trypsin by Mellanby and Woolley. I further suggested that different sets of side chains might be responsible for these different functions.

In a later paper [1914] Mellanby and Woolley take exception to my suggestion and say "Pancreatic rennin and trypsin are identical. In fact the coagulation of milk by trypsin is an expression of a general law that all proteolytic ferments coagulate milk provided sufficient calcium be contained in it." These authors further say "The unique fact that the ferment or ferments in pancreatic juice which digest protein and coagulate milk should withstand boiling in acid solution is practically conclusive proof that the two actions are produced by one and the same substance." If this assumption of Mellanby and Woolley is correct, however, then the milk coagulating power and the power to hydrolyse both fibrin and caseinogen should presumably be quite parallel in their behaviour. The experiments detailed in the present paper, and those described previously [Edie, 1919], however, tend to show that the digestion of fibrin and of caseinogen, if carried out by one enzyme, involves at least two sets of groups of the enzyme molecule, and therefore cannot be said really to be produced by the same substance in the sense evidently meant by Mellanby and Woolley.

I have also carried out some experiments comparing the milk coagulating power of pancreatic extracts with their proteolytic power, and shall now deal with these.

25. To 20 cc. of pancreatic extract (alcoholic) was added 0·5 cc. *N* HCl. Half of this was then heated to 100° for 1 minute and filtered.

1 g. fibrin. Digestion 2·75 hours at 37°.
(*a*) 1 cc. trypsin, 40 cc. 0·5 % Na_2CO_3. Digestion 21·0 cc. *N*/10 nitrogen.
(*b*) 1 cc. trypsin (heated), 40 cc. 0·5 % Na_2CO_3. Digestion 10·2 cc. *N*/10 nitrogen.
To 20 cc. milk was added 1 cc. of trypsin (1) fresh trypsin.
" " " " " (2) heated trypsin.
(1) Complete coagulation in 6 minutes at 37°.
(2) No coagulation in 3 hours.

26. Similar to last experiment.

1 g. fibrin. Digestion 2·5 hours.
(*a*) 1 cc. trypsin, 40 cc. 0·5 % Na_2CO_3. Digestion 26·2 cc.
(*b*) 1 cc. trypsin (heated), 40 cc. 0·5 % Na_2CO_3. Digestion 8·1 cc.
(1) 20 cc. milk, 1 cc. fresh trypsin. Complete coagulation in 5 minutes.
(2) 20 cc. milk, 1 cc. heated trypsin. No coagulation in 2 hours.

From these two experiments it will be seen that though the heated trypsin is still able to digest a considerable amount of fibrin, its milk coagulating power, if any, is now quite negligible.

27. 20 cc. trypsin + 0·5 cc. *N* HCl. Half kept at 100° for 1 minute and filtered.
    (*a*) 1 cc. trypsin, 20 cc. milk. Digestion 39·3 cc.
    (*b*) 1 cc. trypsin (heated), 20 cc. milk. Digestion 4·6 cc.
    Digestion 1 hour. Tannic acid added and digestion estimated as in the usual caseinogen experiments.
    (1) 20 cc. milk, 1 cc. fresh trypsin. Complete coagulation in 5 minutes.
    (2) 20 cc. milk, 1 cc. heated trypsin. No coagulation in 2 hours.

28. Similar to last experiment.
    (*a*) 1 cc. trypsin, 20 cc. milk. Digestion 38·4 cc.
    (*b*) 1 cc. trypsin (heated), 20 cc. milk. Digestion 3·9 cc.
    (1) 20 cc. milk, 1 cc. fresh trypsin. Complete coagulation in 5 minutes.
    (2) 20 cc. milk, 1 cc. heated trypsin. No coagulation in 2 hours.

These two experiments confirm Nos. 25 and 26 in showing that the milk coagulating power of pancreatic extracts is more readily destroyed by heat than the proteolytic power.

It was noticed that the coagulated casein gradually dissolved under the influence of the fresh trypsin, digestion of this protein taking place rapidly even in neutral solution.

More striking differences are found between the milk coagulating power and the proteolytic action of pancreatic extracts under certain conditions without subjecting these to any such drastic treatment as heating to 100° involves. Edkins [1891] found that fresh, active pancreatic extracts were not so active in altering milk so as to produce Roberts' "metacasein" reaction as were older extracts, but that the proteolytic action was greater in the fresh extracts. Edkins suggested that the production of the metacasein reaction might be an aspect of the proteolytic enzyme of the pancreas. Halliburton and Brodie [1896] confirmed what had been pointed out by Benger, that freshly prepared extract of pig's pancreas had very little curdling action on milk, but acquired this property on being kept a considerable time. They accounted for this fact by supposing that the trypsin at first masks or hinders the milk curdling enzyme, but that the former enzyme deteriorates more quickly and so finally allows the rennin to reveal its presence. Vernon [1901] obtained similar results and found that the ratio of rennin value to tryptic value varied largely in different extracts of pancreas and also in the same extracts at different times. In alcoholic extracts the ratio usually became higher as the extract became older, the tryptic value deteriorating more rapidly than the rennin. In glycerol extracts, however, the ratio diminished after say nine weeks, owing to the trypsin being liberated more slowly from its zymogen than the rennin. Some glycerol extracts which were very rich in trypsin gave practically no clot at all, as though the clot were dissolved nearly as fast as it was formed. In a later paper, Vernon [1903] found that rennin and trypsin were precipitated from pancreatic extracts to practically

the same extent when excess of alcohol was added. He considered that in the case of trypsin some groups have the property of coagulating milk and others have the proteolytic power.

I have tested a number of pancreatic extracts at different stages and record some of the principal results below.

In each case sheep's pancreas was used. It was finely minced and extracted with two and a half times its weight of water and a little chloroform. The experiments were similar to Nos. 25 to 28 in technique.

29. Pancreas extracted for two days and extract then tested.
1 cc. trypsin, 40 cc. 0·5 % Na_2CO_3, 1 g. fibrin. 14·9 cc. digested in 3 hours.
1 cc. trypsin, 40 cc. caseinogen (1·5 % in 0·5 % Na_2CO_3). 44·6 cc. digested in 1 hour.
1 cc. trypsin, 40 cc. milk. 23·3 cc. digested in 0·5 hour.
1 cc. trypsin + 20 cc. milk. No coagulation in 0·5 hour. No coagulation on now adding an active coagulating extract, as the caseinogen had been changed (as can be seen above) into products which no longer give a coagulum with rennin.

30. Pancreas extracted for 1·5 hours and extract then tested.
26·4 cc. fibrin digested in 3 hours.
41·8 cc. caseinogen digested in 1 hour.
No coagulation of milk, but much digestion (tannic acid).

31. Pancreas extracted for 3 days and extract then tested.
26·7 cc. caseinogen (milk used) digested in 0·5 hour.
No coagulation of milk.

32. Pancreas extracted for 3 days and extract then tested.
31·0 cc. caseinogen digested in 0·5 hour.
No coagulation of milk, but much digestion.

These four experiments show that freshly prepared aqueous extracts of pancreas generally do not coagulate milk, but are very active proteolytic agents, both on fibrin and on caseinogen.

In a few cases I have found that the filtrate after three hours' extraction coagulated milk rapidly, but this was exceptional.

It has already been mentioned that Halliburton and Brodie accounted for the fact that fresh extracts have very little curdling action on milk by supposing that trypsin deteriorates more quickly and in a few days or weeks allows the rennin to reveal its presence. Vernon seems to suggest that a clot is formed but is dissolved almost at once. In the extracts which I used, the coagulating power seemed to be fully developed within 15 days, but I never found the proteolytic power to diminish as rapidly as would have to be the case if the above suggestion accounted for all the facts.

The extract used in experiment 30 was tested again for proteolytic power when five weeks old. Complete coagulation of milk now took place within four minutes, and the amount of caseinogen digested was now 44·7 cc., this being actually slightly more than it digested at first. If in the first case a clot were formed but almost immediately redissolved, this should have been still more the case when the proteolytic power had increased, instead of which complete coagulation rapidly took place. In other cases the milk coagulating power seemed to have developed completely within four days, the proteolytic

power being practically the same as at first and there was no evidence whatever in support of the view that with extracts a few hours old coagulation really takes place but the clot is redissolved almost instantly. These last experiments once more show that pancreatic extracts differ greatly in their milk coagulating and proteolytic powers. This again points to there being either two or more separate enzymes present, which develop at very different rates from their zymogens, or at least the groups which are responsible for the different functions develop their properties quite independently.

Summary.

1. The amount of acid required in order to protect trypsin from destruction by heat depends on the amount of protein present. The more protein in solution, the more acid required. If not enough acid is present to afford complete protection to the trypsin, the fibrin digesting power is usually destroyed by heat to a considerably greater extent than the power to digest caseinogen.

2. Hydrochloric acid at moderate temperatures also destroys the fibrin digesting power considerably more rapidly than the caseinogen digesting power.

3. The relative amounts of fibrin and caseinogen digested vary very much in different pancreatic extracts.

4. The milk coagulating power of pancreatic extracts is more easily destroyed by heat than the proteolytic power.

5. Generally, but not always, freshly prepared pancreatic extracts have no milk coagulating power. These extracts are always actively proteolytic, but the proteolytic power does not fall off so rapidly as to justify the assumption that the non-appearance of a coagulum with milk is due to the coagulum being really formed but instantly redissolved. All these facts point to the proteolytic and milk coagulating powers of pancreatic extracts being due to a number of distinct enzymes, or, if only one enzyme is concerned, to the different functions being due to different groups of the molecule.

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LX. A NOTE ON THE QUESTION OF THE IDENTITY OF GASTRIC RENNIN AND PEPSIN.

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Much has been written on the question of the identity of gastric rennin and pepsin, and two theories have been brought forward. One, first associated with Pavlov [Pavlov and Parastschuk, 1904], is that pepsin and rennin are identical, Savjalov [1905] and Gewin [1907] holding that coagulation is the first stage in the digestion of milk by pepsin. The identity theory is based on the parallelism between the behaviour of the proteolytic and milk coagulating actions of gastric extracts under different conditions. The other theory is that the enzymes are different. There are two possibilities here, one, put forward by Nencki and Sieber [1901] and others, being that "pepsin" consists of a large molecule with different side chains, one set of which digests protein in acid solution, while another set is responsible for the coagulation of milk in neutral solution. A second possibility is that there are two distinct enzymes involved in the two functions. This is the theory which has been mainly developed by Hammarsten [1908]. It is difficult to distinguish experimentally between these two possibilities, and indeed the present state of our knowledge of the constitution of enzymes renders a distinction hardly practicable. Both depend on the fact that by suitable treatment the two actions can be separated from one another, so that a solution may be obtained which coagulates milk but has no proteolytic action, and on the other hand it is also possible to obtain an active proteolytic solution which has no milk coagulating properties.

Porter [1911] in support of Hammarsten's theory found that various commercial preparations, while coagulating milk, were actually anti-peptic. A full discussion of the subject is given by Oppenheimer [1913].

When investigating the development of enzymes from foetal life onwards I tested the properties of extracts of stomachs of young rabbits and compared these with similar extracts from adult animals. The differences found are recorded in this note.

According to Oppenheimer, pepsin is already present in the stomach of rabbits before birth, while according to Gmelin [1902] rennin is absent from the stomach of new-born animals. Other observers find that rennin develops

very rapidly after birth. Rakoczy [1910, 1911] and Van Hasselt [1910] found that in the case of calves the rennin disappeared rapidly during the first month after birth, while the pepsin increased greatly. The youngest animal employed by Rakoczy was apparently nine days old, but Van Hasselt does not state the exact age of the animals he used. In my experiments the stomachs of rabbits were taken as soon as possible after birth, washed out thoroughly and ground up with twice their weight of water. A little chloroform was added and after three days the liquid was filtered off and the filtrate tested. The stomachs of adult rabbits were tested in exactly the same way. The extracts were subjected to no further treatment with acid, sodium chloride or other substances such as were used by Hammarsten and others with a view to destroying the proteolytic or milk coagulating action as the case might be.

In the coagulating experiments 1 cc. of extract together with 5 cc. of milk was kept in a water-bath at 37° and examined every five minutes. Coagulation was considered complete when the test tube could be inverted without disturbance of the contents. Experiments were always repeated three times at least.

The proteolytic experiments were carried out on fibrin which had been finely minced, thoroughly washed and heated to 85°. 1 cc. extract + 40 cc. $N/20$ HCl + 1 g. fibrin were kept at 37° for a certain time and then filtered, the nitrogen being determined in the filtrate by Kjeldahl's method. Controls (HCl + fibrin) were also done and allowance was made for the amount of nitrogen originally present in the extracts.

The results are expressed as the number of cc. of decinormal nitrogen obtained from the fibrin digested.

Results of experiments:

A. New-born rabbits (each represents a different litter):

No.	Fibrin digested in 2 hours	Coagulation time
1	0·0 cc.	12 minutes
2	,,	18 ,,
3	,,	30 ,,
4	,,	12 ,,
5	,,	13 ,,
6	,,	22 ,,

B. Adult rabbits:

No.	Fibrin digested in 1 hour	Coagulation time
1	4·6 cc.	No coagulation in 2 hours
2	8·0	,, ,, 2 ,,
3	9·3	,, ,, 1 hour
4	23·8	,, ,, 2 hours
5	8·4	,, ,, 2 ,,

These results show the very wide differences which exist between the gastric extracts prepared from young and adult rabbits respectively with regard to their content of rennin and pepsin.

In some cases the extract from young rabbits' stomachs was incubated with acid and fibrin for 18 hours without any appreciable amount of digestion taking place.

At the end of every coagulation experiment, in the case of the adult extracts, a few drops of active rennin were added to the mixture of extract + milk. Coagulation now always took place, proving, if need be, that the absence of coagulation at first was not due to lack of calcium or other deficiency in the milk, but to lack of rennin in the extract. These facts, so far as they go, seem to argue against pepsin and rennin being identical. The extracts were exactly similar in mode of preparation and it is unlikely that one set would contain any inhibitory substance (for example removable by dialysis) which would be absent from the other. No stomach, either of young or adult rabbit, was an exception to the rule that in young animals we get rennin but no pepsin, and as the animal reaches adult life the rennin entirely disappears but pepsin is now found in the stomach.

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LXI. THE ESTIMATION OF NON-PROTEIN NITROGEN IN BLOOD BY A MICRO-KJELDAHL METHOD.

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The following description shows the application of a micro-Kjeldahl method to the estimation of non-protein nitrogen in blood; the same method could, of course, be used for other nitrogen estimations. The procedure given below is recommended on account of its simplicity; some methods which are described have reached a stage of complexity such that they are scarcely practicable in clinical work. Attention is directed to (1) the use of a metal bath in combustion, (2) the method of making the fluid alkaline after combustion. The difficulty in estimations on alcoholic extracts of blood is that the fatty compounds are very resistant to combustion; in estimations on fat-free materials, *e.g.* urea solutions or urine, a paraffin bath at 180–190° can be used in place of the metal bath.

Mix 5 cc. serum with 25 cc. methylated spirit in a flask, which is then corked and allowed to stand for a time. Filter and place two portions of 10 cc. in large test-tubes (8 × 1 inch; *A* in Fig. 1); add 4 drops caprylic alcohol and 2 drops nitrogen-free sulphuric acid. Place the test-tubes as nearly vertical as possible in a beaker of water; keep the water boiling until the alcohol is evaporated and the residue begins to blacken. Throughout the process of combustion it is very important to avoid as far as possible the spread of unoxidised material up the sides of the tube. Add 3 cc. of the following "sulphate mixture":

Water	200 cc.
Nitrogen-free sulphuric acid ...	100 cc.
Potassium sulphate	21 g.
Copper sulphate	5 g.

(Some potassium sulphate crystallises out when the mixture cools.)

Hold the test-tube in a paper holder and boil until the water is evaporated; rapid shaking is necessary to prevent bumping. When the liquid thickens and chars and acid fumes appear, lay the tube in a metal bath ("compo" tubing heated over a Bunsen burner in an enamelled bowl). If all water has not been driven off, the tube is apt to crack when laid on the molten metal. The tube is left in the bath for 15 minutes or so, and then examined for unoxidised material on the sides. This may be removed (1) by very vigorous

boiling of the acid over a free flame; the condensing acid vapours then wash down the sides of the tube; (2) by tilting the tube so that the hot acid runs nearly up to the brim; (3) by washing down with a little water when cool; this last is not a very effectual method. Tubes must always be heated thoroughly over a free flame before being placed on the metal bath. When combustion is complete the tube is allowed to cool, and 8–10 cc. water are added.

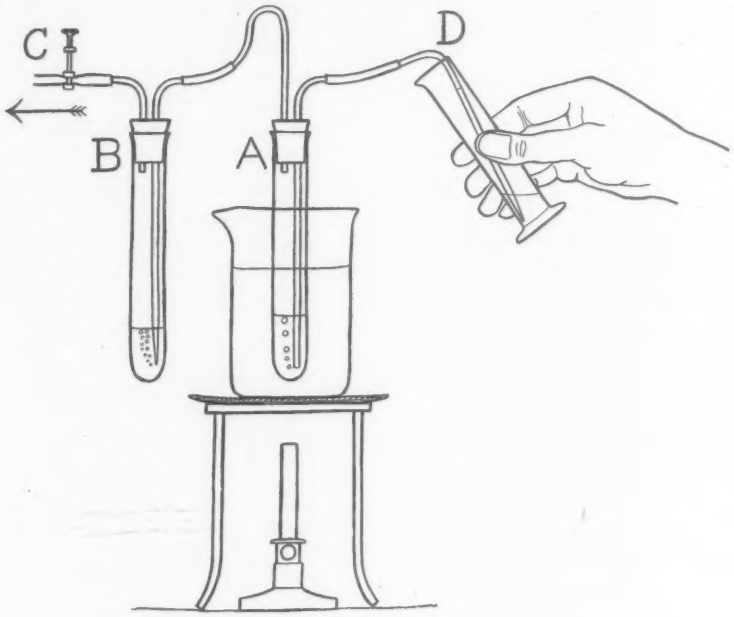

Fig. 1.

The distillation apparatus is shown in Fig. 1, and need not be described in detail; no rubber discs, perforated tubes, bulbs filled with wool, special burners, or condensers, are used. If sufficient water pressure is available, two sets of apparatus containing the duplicate samples are connected to one pump by means of a Y-piece. Two test-tubes (*A* and *B*) of size 8 × 1 inch are held in position by clamps which are not shown in the figure. The tube entering *B* is drawn to a not very fine point; the tube leaving *B* is connected to a water pump fitted with a safety bottle and, preferably, with a mercury manometer. *B* contains 15 cc. 0·01 *N* H_2SO_4 and seven drops 0·2 % methyl red in alcohol.

When the tubes are in position, screw up clamp *C*, turn the pump full on, and loosen the clamp until a gentle stream of air is drawn through apparatus. A glass tube *D* is connected by rubber tubing to the inlet tube of *A*, and dipped into 10 cc. 40 % caustic soda in a cylinder; the soda is thus drawn

slowly into the diluted acid. This stage is shown in the figure. As the last of the soda is drawn in, and a stream of air follows, the two fluids become mixed thoroughly and the deep blue colour of cupric hydroxide appears showing that excess of alkali is present. The tubes by which the soda was introduced are then removed and a wash-bottle of dilute sulphuric acid connected up in their place to remove ammonia from the incoming air.

Air is drawn through, at first quite gently, but towards the end as rapidly as can be done without danger of frothing over. With the pump used in these experiments half-an-hour's duration of air current sufficed for removal of all ammonia. The beaker around test-tube *A* contains water which during the half-hour is heated to boiling. It is especially to be noted that the "condenser" *B* becomes too hot to be held; this is an advantage for the rates of diffusion and combination of the ammonia are thus increased. It was found impossible to recover these small amounts of ammonia quantitatively until heating was adopted. At the close, the rubber tubes on each side of *B* are detached before the pump is stopped; the tube entering *B* is washed down, and the acid titrated with 0·01 *N* NaOH. The whole estimation can be carried out in two hours. A "blank" estimation on the reagents gives from 0·2 to 0·35 cc. 0·01 *N* alkali. Estimations on sera sent for the Wassermann test have shown from 30 to 50 mg. N in 100 cc., which is the range found in whole blood by other workers.

TESTS OF METHOD.

(1) *Recovery of ammonia*, without combustion:

(*a*) 5 cc. ammonia solution added direct to 15 cc. 0·01 *N* acid ... ... 7·10, 7·25, 7·10, 7·15, 7·10, 7·10, 7·10
Mean ... 7·13

(*b*) 5 cc. ammonia solution + 3 cc. "sulphate mixture"; recovered by distillation ... ... 7·20, 7·20, 7·20, 7·05, 7·20
Mean ... 7·17

(*c*) Blanks ... ... 0·2, 0·05, 0·1, 0·1, 0·15, 0·1, 0·15
Mean ... 0·12

Mean cc. 0·01 *N* found ... 7·17
Less Blank ... 0·12

7·05 = 98·9 % of 7·13

(2) *Combustion of urea:*

0·1472 g. urea in 250 cc.
5 cc., calculated ... ... 9·80
5 cc., by Kjeldahl ... ... 9·95, 10·10, 9·80, 10·2, 10·15, 10·0, 9·80
Mean ... 10·00
Less Blank ... 0·325

9·675 = 98·7 %

(3) *Combustion of alcoholic extracts of sera:*

Example:
15 cc. extract of serum H ... ... 10·1, 10·25, 10·25
10 cc. „ „ ... ... 6·9, 6·9, 7·0, 6·95, 6·8, 6·9
Mean ... 6·91
$\frac{3}{2}$ of 6·91 ... 10·36

LXII. DENITRIFICATION AS A MEANS OF SEWAGE PURIFICATION.

By EVELYN ASHLEY COOPER.

From the University of Birmingham.

(*Received July 14th, 1921.*)

The process of denitrification is of importance not only in agriculture, but also in the problem of sewage disposal, because it is capable of effecting purification to a considerable degree. It has been recognised for some time that the presence of nitrates in an effluent is desirable, because, should the dissolved oxygen be expended locally by fermentation of organic matter in a river, the supply of nitrates is then called upon to destroy some of the fermentable matter, and a possible nuisance is prevented.

It is known that the process of denitrification is due to the presence in the sewage of micro-organisms. Many different species can effect the reduction of nitrates, the products of the reaction depending on the kind of organism, *e.g.* nitrogen, nitrites, oxides of nitrogen, ammonia. *B. coli communis*, for example, forms nitrites. The reaction consists in the oxidation of carbonaceous matter by nitrate through the agency of the denitrifying organisms and can be represented thus:

$$5C_6H_{12}O_6 + 24NaNO_3 = 24NaHCO_3 + 6CO_2 + 18H_2O + 12N_2:$$

and it has accordingly been found that when a filter effluent containing nitrates is added to sewage, there is a rapid disappearance of the nitrate. At the present time the nitrate from sewage purification plants is discharged into the rivers, and although it may there act as reserve oxidising material, it undoubtedly encourages the growth of aquatic weeds.

The loss of nitrate in this way is very wasteful, as it could be much more usefully employed in oxidising fermentable material present in the sewage and thus contribute to the process of sewage purification. This could be effected by passing a portion, *e.g.* one-half of the sedimentation tank liquor through filters, and then mixing the filter effluent which would now contain nitrates with the remaining portion of tank liquor. Denitrification would take place, and some of the oxidisable material present in the sewage would then be destroyed. In this way an economy might be introduced, as a smaller area of filtering material would be sufficient for purifying the sewage.

The experimental work described in this paper aims at determining the degree of purification that can be effected in the process of denitrification.

1. Experiments were first of all carried out to ascertain to what extent nitrates could be reduced by the oxidisable constituents of sewage.

For this purpose sedimentation tank liquor was mixed with an equal volume of a solution of potassium nitrate or a filter effluent. The mixtures were kept in stoppered bottles at 20° C. for varying periods, and the amounts of nitrate present before and after the experiment were determined by the colorimetric phenolsulphonic acid method.

Nitrates. Parts per 100,000 *(as N).*

	Original mixture	After 24 hours	After 48 hours	After 96 hours
1. Tank liquor + distilled water	0·00	0·00	—	0·00
Tank liquor + KNO_3 solution	2·50	1·28	—	Trace
Tank liquor + KNO_3 solution	5·00	3·10	—	2·50
2. Tank liquor + KNO_3 solution	2·50	0·10	—	—
	5·00	0·00	—	—
	10·00	0·00	—	—
3. Tank liquor + KNO_3 solution	2·50	—	Absent	—
	5·00	—	1·00	—
4. Filter effluents alone	4·76	1·00	—	—
	16·66	3·33	—	—
5. Tank liquor + KNO_3 solution	5·00	—	1·66	—
	2·50	—	0·00	—
6. Tank liquor + filter effluent	1·50	—	Trace	—
	1·25	—	Trace	—
7. Tank liquor + KNO_3 solution	2·50	—	0·75	—
	5·00	—	3·33	—

A survey of the tabulated results shows that sewage constituents are able to reduce considerable amounts of nitrate in a comparatively short time. Thus, in one experiment 10 parts per 100,000 of nitrate disappeared completely in 24 hours. In another case, a concentration of 16·66 parts was reduced to 3·33 in the same time.

Mixture	Period of preliminary incubation	Pts. per 100,000 Nitrates present in mixture		Pts. per 100,000 "Dissolved oxygen absorbed" by mixture after the preliminary incubation		
		Before	After	1 day	3 days	5 days
1. Tank liquor and water	48 hours	0·00	0·00	3·44	7·00	8·92
Tank liquor and nitrate solution	,,	5·00	1·66	0·60	3·46	4·46
,, ,,	,,	2·50	0·00	1·34	3·44	7·20
2. Tank liquor and water	,,	0·00	0·00	—	—	2·90
,, ,, filter effluent	,,	1·25	Trace	—	—	2·07
Filter effluent and water	,,	1·25	1·11	—	—	0·50
3. Tank liquor and water	,,	0·00	0·00	—	—	5·59
,, ,, nitrate solution	,,	2·50	0·75	—	—	0·92
,, ,, ,, ,,	,,	5·00	3·33	—	—	1·14
4. Tank liquor and water	,,	0·00	0·00	—	—	4·66
,, ,, filter effluent	,,	1·50	Trace	—	—	3·85
Filter effluent and water	,,	1·50	1·33	—	—	0·20

2. Experiments were next carried out to ascertain to what extent the purification of sewage could be effected through the utilisation of nitrates

by the reducing micro-organisms present in the sewage. For this purpose a series of quantitative experiments were set up in which mixtures of (1) sedimentation tank liquor and an equal volume of water, and (2) sedimentation tank liquor and an equal volume of nitrate solution or filter effluent were incubated at 20° C. in closed bottles for definite periods, *e.g.* 48 hours. At the end of this period the "dissolved oxygen absorbed in 5 days[1]" (or sometimes in shorter periods) was then estimated for each mixture.

When filter effluents were used, the "dissolved oxygen absorbed in 5 days" figures were also determined after dilution with an equal volume of water and incubation for two days.

The results are tabulated above.

Conclusion.

The results show that the dissolved oxygen absorption figures for the mixtures of tank liquors and nitrate solutions or filter effluents are very much lower than those for the corresponding mixture of tank liquor and water only. As the dissolved oxygen absorption is a measure of the amount of oxidisable carbonaceous or putrescible matter present in the sewage, the results indicate that incubation of the sewage with nitrate solutions and filter effluents leads to the destruction of a considerable amount of this oxidisable material. With concentrations of nitrate amounting to about 5 in 100,000 it is seen that the degree of purification is enormous, while with lower concentrations, *e.g.* 1·25 parts, the purification is still appreciable.

Another point emerging from the results is that the extent of destruction of the oxidisable matter is not necessarily proportional to the amount of nitrate present.

In one of the experiments (1) the dissolved oxygen absorbed in one day, three days and five days was estimated. A comparison of this series of results reveals the fact that the one day tests indicated a higher degree of purification through denitrification than the three and five days tests. From this it is concluded that the very readily fermentable matter is first attacked in the process of denitrification, and that the more resistant oxidisable matter is largely left and its presence is indicated in the prolonged dissolved oxygen tests.

As it is this readily oxidisable material that causes the nuisance in river pollution, the value of employing the nitrates produced in the filters for further sewage purification is self-evident. In fact, the dissolved oxygen tests summarised above justify the view that in practice considerable economy could be introduced in sewage purification by mixing part of the sedimentation tank liquor with the filter effluent and thus employing the nitrate present for destruction of the more readily fermentable material. By this means a smaller filtering area would be sufficient to effect sewage purification.

[1] Winkler's method was employed for estimating dissolved oxygen.

LXIII. A METHOD OF PROTEIN REMOVAL FROM BODY FLUIDS FOR THE PURPOSE OF SIMULTANEOUS DETERMINATION OF MANY CONSTITUENTS.

By GENKO MUKAI.

From the 3rd Medical Clinic of the Imperial University of Kiushiyu, Japan.

(*Received July 12th, 1921.*)

The chemical analysis of blood involves the use of some method of protein removal. The necessity arises very frequently in practice to estimate many substances simultaneously in the same sample of blood, which necessitates much time and is very inconvenient if the proteins are to be removed for the determination of each substance. I decided, therefore, to seek an accurate and simple method of removing protein without impairing the analysis of almost all the constituents of blood. The method described here, I believe, answered my purpose and was proved to be applicable for the determination of non-protein nitrogen and chlorides in serum, whole blood, other body fluid and urine.

1. Removal of Protein.

In a suitable protein precipitant three properties are desirable for my purpose: (1) the precipitant should be used in a watery solution so as to make a complete extraction of all non-protein constituents soluble in water, (2) at the same time it should not carry down any substances which are required to be examined and (3) it should be free from such substances.

Most of the usual protein precipitants fail to comply with one or more of these conditions. A satisfactory result in the removal of proteins from urine, which I obtained by means of heat coagulation with acetic acid and potassium sodium tartrate, suggested the use of similar solutions. After a number of experiments with several salts—potassium sodium tartrate, sodium oxalate and sodium citrate—it appeared that sodium acetate closely approximated to the ideal precipitant. However there was a slight turbidity by addition of sulphosalicylic acid in the filtrate, which was proved to be protein and metaprotein. Although this quantity varied when the concentration of the reagents was changed, there was always more or less residue of protein. To overcome this difficulty I took advantage of the adsorptive capacity of talcum powder, which readily removed the last trace of protein and metaprotein from this fluid.

The method. Solutions required:

(1) Acid sodium acetate solution.

A hot 20 % watery sodium acetate solution is acidified with acetic acid until its reaction becomes acid to alizarin red.

(2) Dilute acetic acid (1 %).

The technique of the method:

5 cc. of serum[1] is measured into a beaker containing about 80 cc. of distilled water, and then is neutralised with dilute acetic acid using litmus paper as an indicator. The fluid is heated quickly over a small flame just to boiling. During heating the fluid should be stirred gently with a glass mixer to avoid sudden bubbling. 10 cc. of acid sodium acetate solution is at once added to the hot liquid and this is allowed to boil about 30 seconds so as to produce a visible coagulum. After cooling, the fluid is transferred into a 100 cc. volumetric flask and the volume is completed with distilled water. After shaking well, the fluid is poured again into the same beaker and about 3 gm. of talcum powder are added to the fluid. The solution is stirred thoroughly for about 5 to 10 minutes and then it is warmed on a water-bath up to 40°–45° C. and mixed well. After cooling, 2–3 gm. of talcum are added and are mixed thoroughly to make adsorption complete and the solution is then filtered. This filtrate can be used for the determination of the various constituents of serum.

II. Accuracy of the method.

The exactness of the protein removal depends entirely on the care with which the operation is made.

Sometimes error is caused by acidifying the fluid too much instead of neutralising before heating, especially for whole blood. Sometimes it is caused by omitting to warm the fluid after the first mixing with talcum. Both must be carefully done to get exact results.

If these precautions are taken, the filtrate is obtained free from proteins.

The tests of (1) Heller, (2) ferrocyanic acid, (3) sulphosalicylic acid and (4) trichloracetic acid were made in the filtrate as well as in the concentrated filtrate with negative results.

It remains to prove that this procedure causes no change in the quantity of substances which are required to be examined. Among many constituents of blood I chose non-protein nitrogen and chlorides for example and examined the efficacy of the method in the determination of such substances in serum.

The accuracy was tested as follows:

(1) All reagents were proved free from such substances as nitrogen and chlorides.

[1] If it is desirable to remove proteins from whole blood, ascitic, pleural fluid and urine, it is necessary to dilute with distilled water so that the concentration of proteins becomes about 0·3 %.

(2) As urine contains just the same non-protein substances as blood, I made some experiments with urine in order to prove that this procedure did not produce any change in the quantity of nitrogen and chlorides in the protein-free fluid.

(3) The method was applied to serum and I compared the results obtained by this method with those by the other exact one.

III. Experimental.

(*a*) Experiment with normal protein-free urine.

5 cc. of urine was treated as described and the final volume was made up to 100 cc. with water. The following were determined in 75 cc. of this filtrate:

(1) The total nitrogen by Kjeldahl's method.

(2) The content of chlorine by Volhard's method.

Both results were compared with those obtained by direct estimation.

Table I. *Nitrogen content in urine.*

Sample	Nitrogen content in 5 cc. of urine. mg. Before applying this procedure	After applying	Difference mg.	% of deficit
1	43·54	42·91	−0·63	1·45
2	15·40	15·26	−0·14	0·91
3	45·01	44·66	−0·35	0·8
4	37·17	37·10	−0·07	0·2
5	44·80	44·66	−0·14	0·3
Average			−0·26	0·7

Table II. *Chlorine in urine.*

Sample	Chlorine content in urine (calculated as NaCl) Before applying this procedure. ‰	After applying ‰	Difference ‰
1	8·2	8·2	0
2	7·45	7·4	−0·05
3	7·82	7·8	−0·02
4	8·35	8·39	+0·04
5	9·17	9·2	+0·03
Average			0

Table I shows that this procedure causes a slight deficit of nitrogen. The average deficit is only 0·7 % of the total amount, the maximum being 1·45 %, on a content of 15·4–45·01 mg. of nitrogen. As normal urine contains always some quantity of colloidal nitrogenous substances, it may be possible to get such a result by treating urine in this manner.

From Table II it may be seen that there is no constant change in the amount of chlorine after treatment by this method.

The maximum differences between the results obtained with and without applying this procedure are + 0·04 ‰ and − 0·05 ‰ on a content of 7·45–9·17 ‰, which are apparently within the limits of experimental error.

(*b*) Experiment with blood serum.

5 cc. of serum was treated as described and the proteins were removed. The following were determined in 75 cc. of this filtrate (equivalent to 3·75 cc. of serum):

(1) Nitrogen content by Kjeldahl's method.

(2) Chlorine content by Volhard's method.

As control determination I adopted Folin-Denis' method [1912] for non-protein nitrogen and the fusion method of Salkowski [1877] for chlorine.

Some samples of other body fluids were also examined for their contents of non-protein nitrogen and chlorine.

Table III. *Non-protein nitrogen.*

Sample			Total non-protein N found in 100 cc. of body fluids. mg.		Difference mg.
Fluid	Diag.	No.	By this method	By Folin-Denis' method	
Serum	Normal man	1	18·6	18·3	+0·3
		2	16·7	15·5	+1·2
		3	19·1	19·5	−0·4
		4	19·3	18·7	+0·6
		Average	18·4	18·0	+0·4
	Nephritis	5	31·8	30·7	+1·1
		6	43·7	41·0	+2·7
		7	38·4	39·1	−0·7
		8	41·1	42·2	−1·1
		Average			+0·5
Pleural fluid	Pleurisy	9	18·7	18·5	+0·2
		10	25·8	25·6	+0·2
		11	19·3	18·5	+0·8
		12	21·7	22·3	−0·6
		Average			+0·2

Table III presents the results of a number of determinations of non-protein nitrogen in serum and pleural fluid by this method as well as by that of Folin-Denis. The results are almost the same, but the value obtained by this method is a little higher. The average difference between them is + 0·4 mg. and + 0·2 mg. respectively in 100 cc. of serum or pleural fluid, the maxima being + 2·7 mg. and − 1·1 mg.

Estimations of chlorides (as NaCl) in serum, pleural fluid and blood were made by this method and by Salkowski's method with the result that an average difference was found between the two methods of only + 0·03 ‰, the maxima being + 0·11 ‰ and − 0·06 ‰, on a content of 5–6 ‰.

Other application. The sugar content in several samples of serum was determined by Bertrand's method after the removal of protein by the method here described.

The result was satisfactory and this method can therefore also be used for the determination of blood sugar.

Table IV. *Chlorine in serum.*

Sample			Chlorine found (calculated as NaCl)		
Fluid	Animal	No.	By this method °/₀₀	By Salkowski's method °/₀₀	Difference °/₀₀
Serum	Man	1	6·05	6·11	−0·06
		2	5·65	5·54	+0·11
		3	5·98	5·92	+0·06
	Ox	4	6·0	6·0	0
		5	5·85	5·84	+0·01
		6	5·76	5·66	+0·1
Pleural fluid	Man	1	5·93	5·85	+0·08
		2	5·70	5·70	0
		3	5·94	5·85	+0·08
Blood	Dog	1	5·4	5·35	+0·05
		2	5·2	5·23	−0·03
		3	5·05	5·05	0
Average					+0·03

SUMMARY.

A method of protein removal is described which is convenient for the simultaneous determination of various constituents of body fluids.

It has been proved by control determinations that this method is applicable for the estimation of non-protein nitrogen and chlorides in blood serum, etc.

I take this opportunity of expressing my thanks to Professor N. Onodera for his kindly advice during this work.

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LXIV. THE ANTI-SCORBUTIC PROPERTIES OF CONCENTRATED FRUIT JUICES. PART IV.

By ARTHUR HARDEN and ROBERT ROBISON.

(*Received July 27th, 1921.*)

In earlier communications [1919, 1920] we have shown that orange juice can be evaporated to a dry residue without suffering any loss of the anti-scorbutic accessory factor, and that the product retains its potency in a very considerable degree after two years' storage in a dry atmosphere at room temperature.

The effect of storage at higher temperatures (29° C.) has now been investigated and, from the results obtained, it is evident that the accessory factor is much more rapidly destroyed under such conditions.

This particular sample of orange juice was dried in December 1919 and was found to afford complete protection from scurvy when fed to guinea-pigs in daily doses of 0·15 g., equivalent to 1·5 cc. of the original juice. The very hygroscopic solid was transferred as rapidly as possible to a screw-top glass jar, which was then placed in a desiccator for a few days before being closed down. In spite of these precautions it is probable that the material absorbed some moisture, for on placing the jar in the incubator, slight softening was seen to take place and the top layer of the substance gradually darkened in colour, owing presumably to oxidation. The jar was kept at a uniform temperature of 29° until February 1921, *i.e.* during 14 months, after which further animal tests were begun. The material was then in the form of a hard toffee-like mass but was found to contain 8 % of water.

A number of guinea-pigs were fed on the usual basal ration of oats, bran and autoclaved milk, together with doses of the dried juice of 0·15 g., 0·3 g., 0·45 g., and 1 g., equivalent to 1·5 cc., 3 cc., 4·5 cc., and 10 cc. of the original juice. All the animals developed scurvy but those receiving the highest dose did not show any symptoms until the 24th day and were still alive on the 63rd day of the experiment. Some measure of protection was therefore given by this amount of the dried juice, but it is evident that more than 85 % of the anti-scorbutic accessory factor had been destroyed during storage under the above conditions. Tests were also carried out with some of the same batch of dried juice that had been stored in a desiccator at room temperature during a similar period, 14 months. Two guinea-pigs, each receiving 0·15 g. daily, showed normal growth for about 50 days but thereafter remained practically stationary in weight. They were killed on the 81st day and symptoms of scurvy were found. A guinea-pig which received a daily ration of 0·3 g.,

equivalent to 3 cc. of juice, showed no signs of scurvy after 81 days, though this animal also failed to exhibit normal growth during the last 20 days of the experiment.

In the experiments previously described it was found that a daily ration of 0·5 g. of dried juice that had been stored during two years at room temperature afforded complete protection from scurvy. It would appear, therefore, that under the conditions detailed above the loss of the anti-scorbutic accessory factor is of the order of 50 % of the amount originally present in the juice.

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LXV. STUDIES ON GELATIN. PART I. THE DYNAMICS OF THE FORMATION OF GELATIN FROM OSSEIN.

By ALEXANDER BERNARD MANNING and SAMUEL BARNETT SCHRYVER.

(*Report to the Adhesives Committee of the Department of Scientific and Industrial Research.*)

From the Biochemical Department, Imperial College of Science and Technology.

(*Received July 21st, 1921.*)

These researches were undertaken with the object of throwing some light on the processes involved in the extraction of gelatin from its precursors in the animal body. In the present communication the results are confined to the investigation of the dynamics of the formation of gelatin from decalcified bones.

Preparation of the materials.

For the purposes of this research, the material used consisted entirely of the middle portions of the femurs of young oxen (about two years old). These were collected from the slaughter-house as soon as possible after the animals were killed, and their preparation for experiment was commenced immediately after they were brought into the laboratory. They were first washed in running water until the washings were clear, and then sawn into two portions along their length, and the marrow and adhering fatty matter was removed. They were then dehydrated and de-fatted, first by treatment with alcohol and then by ether, and afterwards air-dried. They were again cleaned by hand to remove the last portions of adhering tissues. They were then crushed in rock-crushers, for the use of which the authors are indebted to Prof. Truscott. The crushed particles were then sieved, the largest size used passing through $\frac{1}{4}$-inch mesh, but not through a $\frac{3}{16}$-inch mesh; the smallest sized bones used passed a $\frac{1}{32}$-inch mesh. Altogether five grades of bone were decalcified separately, 3 % cold hydrochloric acid (not above 5°) being used for the purpose. The course of the decalcification was followed by the titration of the acid from day to day. This process required from three to seven days, the rate depending on the size of the bone particles. After decalcification, the bones were washed with water till acid-free, then with 1 % ammonia solution, again with water, and finally with alcohol and ether; after which they were dried by a fan.

Method of Experiment.

The rate of extraction of gelatin from decalcified bones of various graded sizes has been measured at 100° and at 90°.

For the extraction at 100° a weighed quantity of the dried decalcified bones was added to a known volume of boiling water in a flask fitted with a reflux condenser. The time was taken, the mixture was kept gently boiling and at convenient intervals a small sample of the liquid was withdrawn and filtered through cotton wool. The nitrogen content of the filtered sample was determined by Kjeldahl's method, and the boiling continued until no further increase in nitrogen content occurred. To measure the rate of extraction at 90° the same method was used except that the flask was immersed in a thermostat at the required temperature and its contents kept in motion by a glass stirrer.

Results.

Fig. 1 shows the results for the extraction of gelatin at 100°. In each case 20 g. of decalcified bones and 300 cc. water were taken. The results are plotted as percentages of the total amount of nitrogen extracted. The total nitrogen extracted varied slightly with different samples of bones, due to differences in degree of dryness. It lay between 0·85 and 0·95 g. per 100 g. solution.

Fig. 2 shows the results of extraction at 90°.

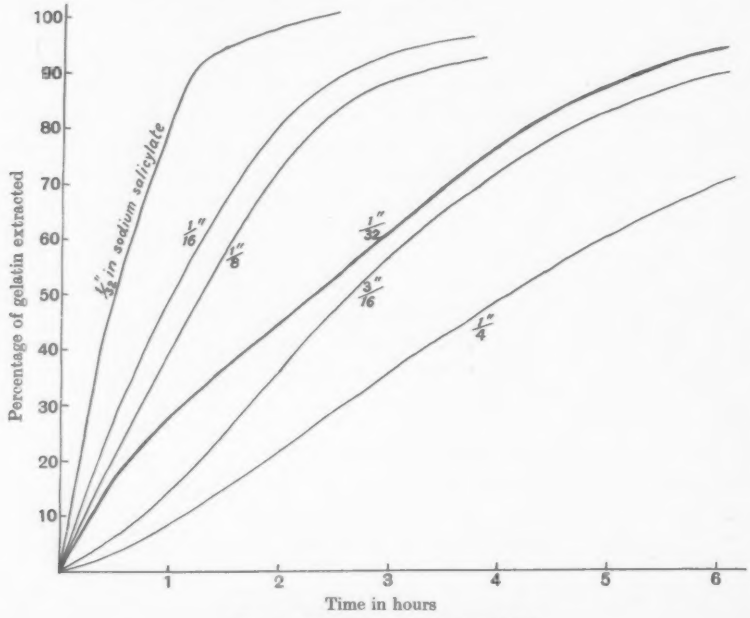

Fig. 1. Extraction of gelatin at 100°.

The extraction curves for the larger sized particles have a point of inflection, and are similar to the curves for autocatalytic reactions. The process of extraction, however, is not autocatalytic. The rate of extraction of a fresh quantity of decalcified bones is unaffected by the presence in solution of some previously extracted gelatin.

The rate of extraction increases with the finer division of the bones, except for the very finest ($\frac{1}{32}$-inch and under). The abnormal curve obtained for the latter size appears to be due to aggregation of the particles. Thus in the presence of sodium salicylate ($N/4$), a salt which markedly lowers the surface tension of water and which would therefore tend to prevent aggregation, the

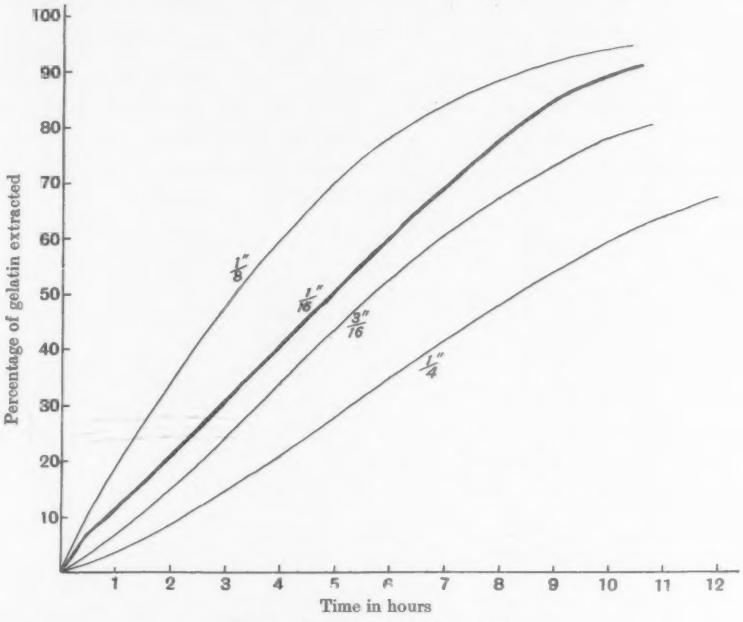

Fig. 2. Extraction of gelatin at 90°.

curve fits normally into the series. A control experiment with $\frac{3}{16}$-inch bones in presence of $N/4$ sodium salicylate showed that the salt had no direct effect on the rate of extraction. The reaction rate was slightly greater than in water, but this could be ascribed to the raising of the boiling point of the water. A similar curve was obtained when the reaction with $\frac{3}{16}$-inch bones was carried out in $N/4$ sodium chloride solution.

In the hope that some light might thereby be thrown on reactions in heterogeneous systems in general, it was decided to investigate further the relation between these curves. Other investigations have shown that ossein is the chief substance remaining after the decalcification of bones, and there

is reason to suppose that this is an anhydride of gelatin. Hoffmeister [1879] showed that when gelatin has been heated to 130° it is not readily soluble in water, but is apparently converted into an anhydride, from which gelatin can be regenerated completely only after boiling for several hours with water. The authors have been able to confirm this observation of Hoffmeister, and have found that the curves representing this regeneration are very similar to those which represent the formation of gelatin from ossein. The details of these experiments are reserved for a subsequent communication.

The extraction of gelatin from bones consists essentially in the hydrolysis of an anhydride and the solution of the gelatin. Apart from the rate of this process itself there are, however, other controlling factors amongst which the initial surface exposed to the action of water plays a very considerable part.

An estimate of the initial surface area of the decalcified bones was made by determining the average number of particles contained in a gram of substance. Assuming that the density is the same throughout and that the shape of the particles of different sizes is similar, the relative surface can be approximately calculated. It is proportional to $\sqrt[3]{n}$ where n = number of pieces to 1 g. In this way the following numbers were obtained:

Size of particles	Surface area (in arbitrary units)
$\frac{1}{4}$-inch	3·75
$\frac{5}{16}$ "	5·71
$\frac{1}{8}$ "	9·65
$\frac{1}{16}$ "	13·75

Plotting these values against the estimated initial rate gives the curves shown in Fig. 3. For the $\frac{1}{8}$-inch and larger sizes the points lie on smooth curves, approximately parabolas. The abnormality of the smaller sizes may probably again be attributed to aggregation.

For the other sizes, $\frac{1}{8}$-inch and greater, we may write:

$$\text{Initial rate} = kS_0^2.$$

This suggests as a general formula for the group of curves:

$$\frac{dx}{dt} = k\{S_0^2 + f(x)\}(a - x)$$

where x = quantity of gelatin extracted;

a = total quantity of gelatin extractable, and

k = constant.

The simplest assumption to make with regard to the form of $f(x)$, which represents the change in the surface effect as the reaction proceeds, is that

$$f(x) = x.$$

This gives

$$\frac{dx}{dt} = k(S_0^2 + x)(a - x)$$

which on integration gives

$$\frac{1}{a + S_0^2} \log \frac{a(S_0^2 + x)}{S_0^2(a - x)} = kt.$$

This represents a group of curves of the type found experimentally. When the initial surface is small the curve has a point of inflection, but beyond a certain critical value, given by $S_0^2 = a$, the point of inflection disappears. With the smaller sizes the curve approaches that of a unimolecular reaction. This would be expected from the equation when S is large compared with x.

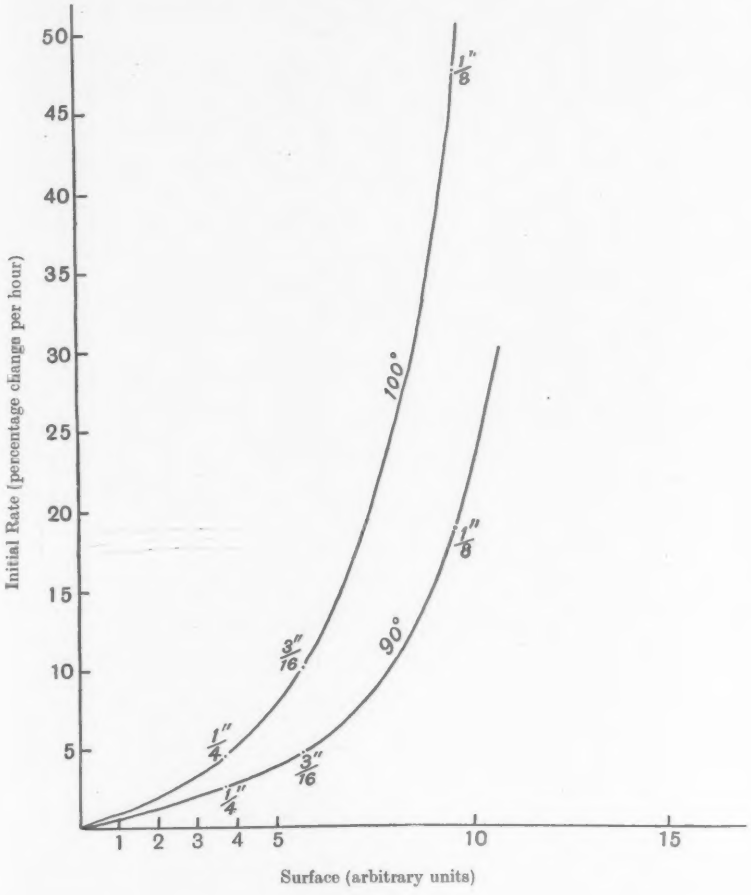

Fig. 3.

Tabulated on p. 528 are corresponding values of x and t taken from the curves and the calculated values for k.

Rate of extraction of gelatin from decalcified bones at 100°.

Size of particles $\frac{1}{4}$-inch $S_0 = 3{\cdot}75$

Time in hours	% extracted	k
1·14	10	·00491
1·90	20	·00510
2·6	30	·00504
3·33	40	·00488
4·13	50	·00468
4·95	60	·00456
7·1	80	·00432

Mean $k = ·00478$

Size of particles $\frac{1}{8}$-inch $S_0 = 9{\cdot}65$

Time in hours	% extracted	k
0·23	10	·00470
0·475	20	·00455
0·74	30	·00445
1·02	40	·00440
1·28	50	·00455
1·58	60	·00464
2·32	80	·00496
3·28	90	·00470

Mean $k = ·00463$

Size of particles $\frac{3}{16}$-inch $S_0 = 5{\cdot}71$

Time in hours	% extracted	k
0·75	10	·00375
1·28	20	·00415
1·73	30	·00440
2·18	40	·00450
2·67	50	·00460
3·50	65	·00460
4·73	80	·00450

Mean $k = ·00436$

Average k for different sized particles = ·00444.

Rate of extraction of gelatin from decalcified bones at 90°.

Size of particles $\frac{1}{4}$-inch $S_0 = 3{\cdot}75$

Time in hours	% extracted	k
2·3	10	·00244
3·9	20	·00248
5·3	30	·00247
6·85	40	·00237
8·4	50	·00230
10·25	60	·00220

Mean $k = ·00238$

Size of particles $\frac{3}{16}$-inch $S_0 = 5{\cdot}71$

Time in hours	% extracted	k
1·5	10	·00187
2·56	20	·00206
3·63	30	·00210
4·65	40	·00212
5·75	50	·00213
7·0	60	·00211

Mean $k = ·00206$

Size of particles $\frac{1}{8}$-inch $S_0 = 9{\cdot}65$

Time in hours	% extracted	k
0·53	10	·00202
1·07	20	·00202
1·74	30	·00189
2·45	40	·00183
3·22	50	·00180
4·05	60	·00181
6·35	80	·00182

Mean $k = ·00189$

Average k for different sized particles = ·00211.

The average temp. coeff. for the interval 90°–100° is 2·1.

In the search for a suitable expression to represent the experimental results an attempt was made to arrive at a more accurate estimate of the initial surface by adsorption experiments. Decalcified bones markedly adsorb iodine and some dyes.

The unexpected result was obtained that the surface was the same independently of the state of division of the bones. The only variation was in

the time required to arrive at equilibrium. This is shown in the curves for iodine adsorption in Fig. 4. In each case 5 g. decalcified bones and 100 cc. $N/50$ iodine were put in a stoppered bottle and shaken. From time to time 10 cc. was withdrawn and titrated with standard thiosulphate. Precisely similar results were obtained with other initial concentrations of iodine and also with diazine-green (a dye of not very great diffusibility and chosen on this account) as adsorbed substance. The conclusion must be drawn therefore

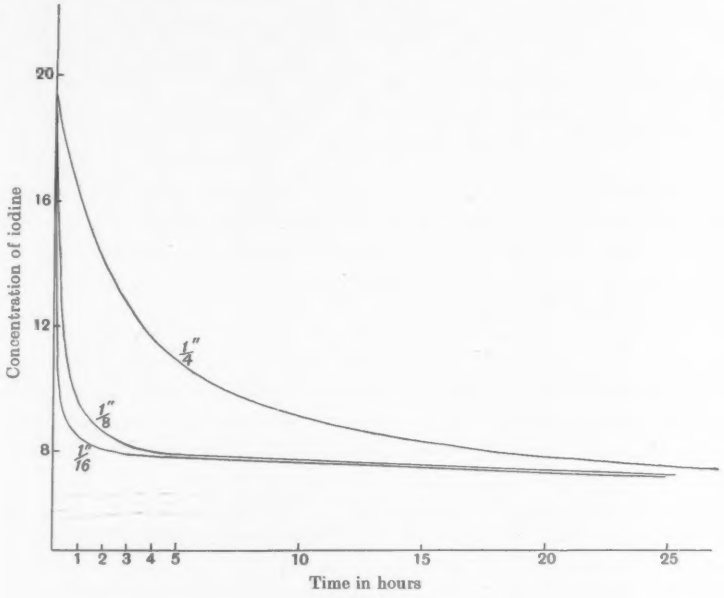

Fig. 4.

that owing to the high porosity of the bone there is the same surface available whatever the size of the particles, and that the different behaviour must be attributed to the time taken for the adsorbed substance to diffuse along the pores and so arrive at the surface.

Similarly in the extraction of gelatin the variation of rate with size of particles is not a true surface effect. The effect is indeed dependent on the apparent surface (original surfaces + fissure surfaces), the dependence being expressed by the formula above. But, except for very small particles, the rate of extraction is controlled largely by the rate at which gelatin diffuses out and water diffuses in through the pores; it is essentially a capillary phenomenon.

REFERENCE.

Hoffmeister (1879). *Zeitsch. physiol. Chem.* **2**, 314.

LXVI. THE FORMATION OF VITAMIN A IN LIVING PLANT TISSUES.

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AND

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(*Received July 29th, 1921.*)

An opinion is gradually being formed that the fat-soluble vitamin occurs in the green, actively assimilating parts of plant tissue and that it is generally absent from the localities where chlorophyll is not found. This led us to carry out certain investigations planned to trace, if possible, the origin of the fat-soluble factor in plants.

Experimental Method.

The essential details of the method of testing have already been outlined in a former paper [Drummond and Coward, 1920, 1]. Throughout this work and in fact now generally, we employ a new method of purifying the constituents of our basal diet from residual traces of the vitamin A. As we have pointed out in the paper referred to above, the chief source of impurity is the caseinogen which persistently carries small amounts of milk fat which may serve as sources of the factor A. Since the discovery that this substance is readily destroyed by oxygenation at high temperatures [Hopkins, 1920; Drummond and Coward, 1920, 2] we have employed a process based on this principle to replace the tedious and expensive method of extraction with alcohol and ether formerly adopted. At the present time we use an oven in which the caseinogen is exposed in shallow layers to air at a temperature of 105° for at least 24 hours, the fine powder being frequently raked over to expose a new surface to the air. We have been entirely satisfied with this simplified method of purifying the basal protein, which yields results quite as reliable as the older method. The foodstuffs to be tested are always given as supplements to each rat before the basal diet is given and in nearly all cases it is consumed before the latter is supplied.

The Vitamin A content of seeds.

The work of McCollum and his co-workers summarised in his recent book [1918] has shown us that seeds generally are poor sources of vitamin A. Our first plan was to test a number of seeds and then to compare the vitamin

content of the plants produced from them at various stages of their development under different conditions. Many experimental difficulties were encountered, however, in consequence of which few systematic tests of any one variety were found possible. Nevertheless, the results of these preliminary tests, even if somewhat incomplete, are given because they are all more or less in agreement with those yielded by the more complete studies.

The seeds first studied were carrot, turnip, cabbage, cress, peas, and white and yellow maize. These were all given to the rats in known amounts daily in addition to the basal diet deficient in vitamin A. No resumption of growth followed the administration of turnip, cabbage or white maize, but there was evidence of more or less activity in the case of the peas, yellow maize and carrot. The rats refused to eat the cress seed nor could they be induced to consume the cress shoots. The average results of these experiments are seen in the table p. 538 (Tests 1–7).

Our observations on the two forms of maize confirm the results recorded by Steenbock and his colleagues [1919 and 1920] and in one case we were able to restore the growth of a rat which had been stationary on the white maize supplement by changing it to one of yellow maize.

It is interesting to note also that sycamore seeds, although their cotyledons are distinctly green, are unable to induce growth even when supplied at the rate of 1·5 g. per day without the fruit wall and wing (Test 8).

On the whole our results confirm McCollum's opinion that the majority of these tissues are deficient in vitamin A. Further evidence is also presented in the experiments on sunflower seeds to be described below.

Germination of seeds.

It was originally shown by Fürst [1912] and confirmed by Chick and Hume [1917] that the anti-scorbutic factor is not present in the dry seeds but is produced during germination. Chick and Delf [1919] state, however, that both dry and germinated peas are deficient in the fat-soluble factor. The latter observation was made during a study of scurvy in the guinea-pig, so we decided to make a direct test on rats, with the result that no detectable increase in the amount of the vitamin A appeared to have occurred in these seeds during germination (Tests 9, 10).

Etiolated seedlings.

The first experiments with seedlings were made with sand cultures. The sand in which the seeds were to grow was well washed in running warm water with much stirring for about half-an-hour. It was then treated with strong nitric acid until no further effervescence of carbonates took place, washed with a 2 % solution of mercuric chloride to kill bacteria and finally washed in a Buchner funnel with distilled water. The sand was spread out in large trays in layers about 1½ inches deep, sprinkled with the seed and a very shallow top layer of sand added. Successive sowings on fresh areas were made

every few days to ensure a continuous supply of seedlings of about the same age. The seeds were watered with distilled water for about a week and then with Sachs' solution for a week, after which Sachs' solution reinforced with 0·3 % of sucrose was employed in the hope that by supplying preformed carbohydrate growth beyond the expansion of the cotyledons might be induced. This hope was not realised and in spite of all our care the etiolated seedlings tended to die when about 3–4 weeks old.

Etiolated seedlings in their prime (generally 13–16 days old) were given to rats in amounts of 0·7 g. per day. Owing to the difficulty of maintaining the supply of sufficient material for testing, the results are not as conclusive as might be desired. No growth was obtained with the carrot seedlings (25–30 days of age) in spite of our having detected some activity in the dry seeds, whilst in the other cases most of the etiolated shoots showed very slight activity (Tests 11–14). Etiolated pea shoots grown from seeds planted in earth and watered with tap water produced some slight increase in weight in the rats (Test 15). As will be shown later, more carefully controlled experiments with sunflower plants indicate that in this species at least, the amount of vitamin A present in the etiolated shoot is not appreciably greater than that in the seed from which it grew.

Green seedlings.

The seeds were treated in exactly the same manner as in the last experiment except that they were exposed to light and only normal Sachs' solution was used for watering. The results of the feeding tests show that green shoots of turnip, maize and peas (soil and sand grown) possess a decidedly higher value as sources of vitamin A than either the seeds from which they sprang or the corresponding etiolated shoots. Abnormal results were obtained in the case of the very young carrot shoots which caused little or no resumption of growth, although fully developed carrot leaves (carrot tops) were more potent (Tests 16–20). Here again we regard these results very much as preliminary in character, and place more reliance on those with sunflower seeds to be described below in which the green shoots were found to be very much more active than the etiolated ones.

Experiments with sunflower plants (*Helianthus annuus*).

The preliminary results briefly outlined above led us to select one species of seed for full investigation and the ease with which the sunflower may be grown under laboratory conditions caused us to choose that plant.

The dry sunflower seeds stripped of the outer husk were found to be relatively inactive as a source of vitamin A when given in daily doses of from one to four seeds per day per rat (Fig. 1). Seeds were germinated in boxes of soil both indoors and in a closely darkened room and after about a fortnight when they had only expanded their cotyledons they were given to the animals as supplements of from one to four shoots daily without inducing

growth (Fig. 2), but vigorous growth followed the administration of a supplement of one or two green shoots daily (Fig. 1). Other seedlings were allowed to produce two further pairs of leaves, and as the cotyledons withered, were tested. The potency of these more mature shoots appeared to be even greater than that of the younger ones (Fig. 2). All attempts to make the etiolated shoots produce further leaves were unsuccessful. It appears reasonable to conclude from these experiments that the formation of the large amounts of vitamin A found in the green leaf of most plants demands the influence of light.

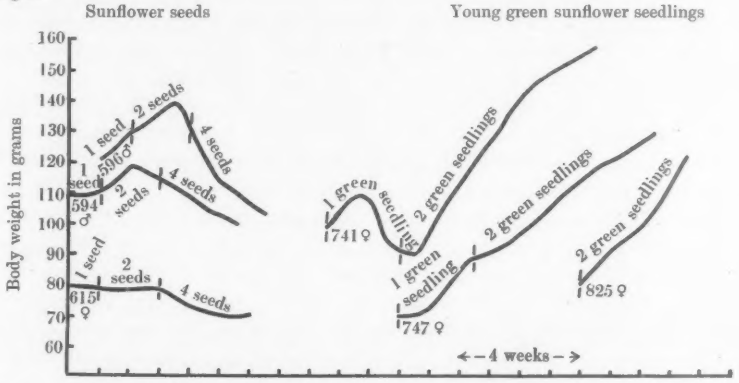

Fig. 1. Curves 594, 596, 615 show failure on sunflower seeds. Curves 741, 747, 825 activity of green seedlings.

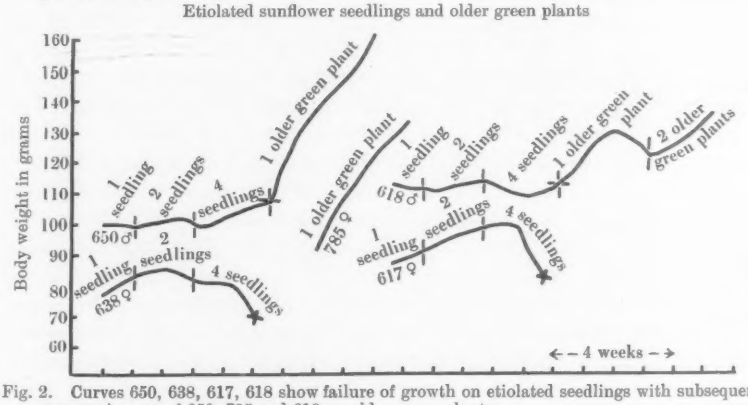

Fig. 2. Curves 650, 638, 617, 618 show failure of growth on etiolated seedlings with subsequent recovery in case of 650, 785 and 618 on older green plants.

WATER-CULTURE EXPERIMENTS WITH *Tradescantia* ("Wandering Jew").

Tradescantia is a plant that lends itself very freely to water-culture experiments and a few tests were made on material obtained in this manner. Preliminary tests showed that shoots of this plant grown in pots could serve as a source of vitamin A for rats (Test 21).

Shoots of about five nodes were taken, cut off sharply immediately below the lowest node, the lowest three leaves stripped off, and the stem placed in a hole in the cork of the culture jar. The stem was padded round with cotton wool and so set that the lowest two nodes were submerged in the culture fluid. Ordinary glass preserving jars of 800 cc. capacity were employed and the cork of each jar carried four cuttings. Sachs' solution was employed as a culture fluid and was renewed every three or four days, the stem and roots of the shoots being at the same time washed and gently brushed in running water. It was found very difficult to keep the cultures free from contamination with moulds, especially in the case of the jars kept in darkness, but the development of green algae in cultures exposed to light was successfully prevented by covering the jars with black cloth up to the level of the fluid.

The shoots kept in the light grew excellently and developed into large sturdy plants in the course of some eight weeks. From the plants, recent shoots were removed and administered to rats in daily doses of 1·5 g. with the result that growth was induced more or less strikingly (Test 22).

That the presence of vitamin A in these shoots did not merely indicate a transference of material from the original cutting is shown by some later experiments in which the same plants were cut down until only half-an-inch of bare stalk remained above the cork. These stumps after a few weeks produced vigorous new shoots with abundant leaves which on testing appeared to possess a value as a source of the vitamin at least equal to that of the original plant.

Trouble was encountered with the cuttings kept in the dark. In the first place they refused to produce new leaves and merely grew by elongation of the internodes, even when the Sachs' solution was reinforced with 0·3 % of sucrose; whilst, secondly, the moulds which frequently appeared in the cultures required constant removal. Testing of these pallid shoots demonstrated that their activity was somewhat less than that of the shoots grown in the light, but it must be remembered that the etiolated shoots were originally green and the loss of chlorophyll may not necessarily have been accompanied by the destruction of the pre-existing vitamin (Test 23).

An attempt was made to grow chlorotic shoots of *Tradescantia* in Sachs' solution devoid of soluble iron salts, but without success. The shoots grown in this fluid appeared to thrive as successfully as those grown in the normal fluid in light and showed no obvious sign of chlorosis. It is possible that the roots were obtaining sufficient iron from the glass jars. These shoots contained at least as much vitamin A as the normal plant (Test 24).

If one is permitted to exclude the complications introduced by the absence of complete sterility in these experiments (and the later results indicate that lower organisms devoid of chlorophyll do not synthesise vitamin A), the production of this substance by the green plant from inorganic sources appears to have been demonstrated from these experiments.

Vitamin A in white and green cabbage leaves.

For a long time the value of green cabbage as a source of the fat-soluble factor has been recognised but a definite comparison of the white and green leaves has not been made. Recently, however, Hume [1921] has reported that growth in guinea-pigs was more satisfactory when green leaves were given than when the white inner leaves were used. In our experiments white and green cabbage leaves respectively were given to rats in quantities of 1·5 g. of the fresh material daily. Practically no growth was obtained with the white leaves but very fair growth was obtained with the green leaves. The quantity corresponded to about 0·24 g. of dry matter per day which appears to be somewhere near the minimum for obtaining growth (Tests 25, 26). Rats which had for some time been stationary on dried white leaves resumed growth when transferred to dried green leaves.

Fat-soluble vitamin in some lower plants.

Mushrooms (*Agaricus campestris*) when fed to rats in rations of 1·5 g. per day induced very slight growth (Test 27). Microscopical examination indicated that the spores tended to pass through the intestinal tract unchanged but that the remainder of the fructification was digested.

Seaweeds. The examination of the marine algae is of very great importance since it would appear that the ultimate origin of the vast supplies of vitamin A which are available in the form of fish oils and liver oils may be represented by these organisms. On two occasions we have tested samples of common green seaweeds found on the south coast of England (*Ulva* and *Cladophora* sp.?) and have found them to be at least as potent as the green land plants such as cabbage (Tests 28, 29).

The examination of a typical brown seaweed, *Fucus vesiculosus*, was impossible since the animals refused to eat it. It is significant that no animal is known to eat this weed.

A red seaweed, *Polysiphonia* sp., induced a slow rate of growth at first, but this was not maintained (Test 30).

The difference in the food value of these weeds may be significant in spite of the small amount of experimental evidence advanced here, since we may recall the relationship between the coloration of these weeds and the activity of the photosynthetic processes at the different depths at which they flourish [see Palladin, 1917; Engelmann, 1883].

Another sample of a red weed, *Chondrus crispus* (Carrageen moss) which had been sun-dried showed no growth-stimulating powers when given in an amount of 0·5 g. per day (Test 31).

The condition of the vitamin A in plant tissues.

Certain observations having led us to suspect that the fat-soluble vitamin might occur in the leaf of green plants in the form of a complex with protein we undertook the following experiments. Preparations of the proteins of

green and white leaves were made by the method described by Chibnall and Schryver [1921]. The fresh leaves were minced and, together with the juice pressed out in the process, allowed to stand in water saturated with ether overnight in the cold. The solid matter was then removed by filtering through a cotton cloth and the residue pressed. Carbon dioxide was passed through the filtrate for 2–3 hours to remove the ether and the solution was heated on the water-bath to 40–50° to precipitate the proteins. The flocculent precipitate was filtered through a folded filter and washed only once with water at which point our method deviated from that employed by Chibnall and Schryver in that further washing to remove substances of a fatty nature was avoided. The residue was spread out on porous tiles in a vacuum desiccator over calcium chloride until practically dry when it was tested on rats in amounts of about 0·1 g. daily, which represented about 40 g. of fresh spinach. Unfortunately, green cabbage leaves were not available at the time in sufficient quantity for a similar test, and tests were made only with protein matter prepared from fresh green spinach and white leaves of cabbage. The results showed a slight activity on the part of the green protein precipitate from the spinach whereas that from the white cabbage was completely inactive (Tests 32 and 33).

The results of these experiments indicate that vitamin A is not present in the form of a complex with protein in green leaves unless the method of preparation was responsible for the resolution of such a complex. The small activity of the preparation from green leaves when given in doses representing so large an amount of the fresh vegetable would suggest that a small amount only of the vitamin had been carried down by adsorption. Chibnall and Schryver mention that substances of a fatty nature can be removed from the crude protein precipitate by extraction with solvents.

Vitamin A in the unsaponifiable matter in green leaves.

It has been shown by Osborne and Mendel [1919] and others that vitamin A can be extracted from green leaves by fat solvents. That it may be obtained in the unsaponifiable fraction of the fatty substances extracted from a typical green plant, alfalfa, by alcohol, was first shown by Steenbock and Boutwell [1920]. This would appear to confirm the original observation of McCollum and Davis [1914] who stated that the vitamin in butter-fat withstood saponification at room temperature in a non-aqueous solution. This view was at first opposed by Drummond [1919] but we have since found that, if care is taken to prevent oxidation, a highly concentrated fraction may be obtained by the cold or the hot saponification of certain animal oils or plant tissues. The previous failure to confirm McCollum's finding is attributed to destruction by oxidation during the process. We have applied this work to a study of certain plant tissues.

Dried Peas. 200 g. of dried peas ("gradus" variety sold for germination) were warmed on a water-bath with a litre of 50 % aqueous potassium hy-

droxide for 14 hours and then allowed to stand for two weeks at room temperature, after which about two volumes of alcohol were added and the saponification completed by boiling under a reflux for 24 hours. The alcohol was removed by distillation *in vacuo*, and the unsaponifiable matter removed by extraction with ether. The subsequent treatment of the ether fraction, washing, drying and concentration by removal of the solvent were conducted in an atmosphere of carbon dioxide. The residue was semi-liquid and was stored in a tube filled with carbon dioxide, and refilled with the gas after each sample had been removed for feeding tests. This fraction was given to rats in a dose of about 0·02 g. corresponding to about 4 g. or 15 peas, daily, and produced definite growth (Test 34). In view of the results previously obtained with the small ration of dried peas themselves, it was decided to ascertain whether a diet containing the amount of dried peas equivalent to the dose of unsaponifiable matter would induce growth. Accordingly a diet consisting of 65 % of dried peas (ground up), 15 % caseinogen, 5 % salt mixture, 5 % yeast extract, and 5 % of lemon juice was tested and found to produce growth.

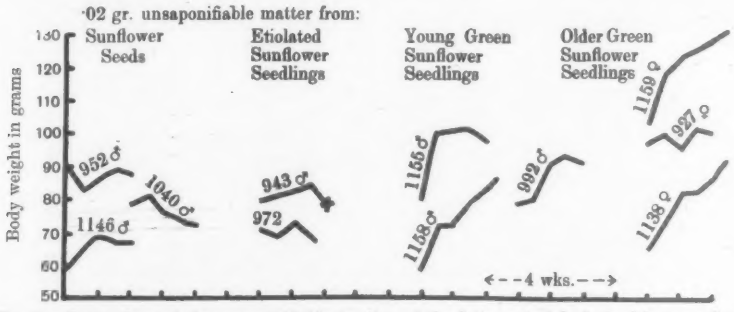

Fig. 3. Comparison of the unsaponifiable fraction of the fatty materials derived from sunflower seeds and from green and etiolated shoots.

Etiolated and green pea shoots were saponified by essentially the same process after having grown for about 20 days in boxes of soil. The shoots only were used, the remains of the cotyledons and the roots being discarded. The crop of etiolated pea shoots, about 425 plants weighing 285 g. yielded very little material but the unsaponifiable fraction when given to rats in daily doses of 0·02 g. appeared to be slightly active. This dose corresponds to 20 g. of fresh shoots. The unsaponifiable matter from the green pea shoots was very small in amount and apparently inactive. For several reasons this test was unsatisfactory, chiefly because of the very small amount of material available (much of it was lost through an accident) and the possibility that in the case of the extract from the green shoot, loss by oxidation might have occurred. Better results however were obtained when the unsaponifiable fractions were prepared from larger quantities of etiolated

and green sunflower shoots. These fractions, prepared by the method we have described, were tested by giving 0·02 g. per day to the rats. As may be seen from Fig. 3, the difference in the growth-promoting power of the material from the two types of shoots is very marked, there being little evidence of the presence of appreciable quantities of the vitamin A in that from the etiolated seedlings.

Typical results of feeding substances as test for fat-soluble vitamin.

Test No.	Substance tested	Daily supplement per rat to basal diet devoid of A	Average weight in g. of rats during test period[1] 0	7	14	21	28 days	Remarks
1	Cabbage seed	0·2 g.	85	88	91	94	88	Very slightly active
2	Turnip seed	0·1 g.	80	78	72	80	77	Inactive
3	Carrot seed	0·4 g.	97	110	115	130	—	Active
4	Dried peas	1 pea seed	93	95	107	100	110	Slightly active
5	„	5 pea seeds	124	129	130	140	—	Slightly active
6	Maize seed (yellow)	1 g.	100	106	109	108	119	Active
7	„ (white)	1 g.	88	87	87	79	80	Inactive
8	Sycamore seed	1·5 g.	112	109	104	105	—	Inactive
9	Germinated peas	1 plant	95	98	105	102	100	Inactive
10	„	4 plants	79	80	86	—	—	Inactive
11	Etiolated cabbage shoots	0·7 g.	60	61	70	72	79	Slightly active
12	„ carrot „	0·7 g.	84	90	87	—	—	Inactive
13	„ turnip „	0·7 g.	68	67	78	85	—	Slightly active
14	„ maize „	1·0 g.	86	90	96	96	—	Slightly active
15	„ pea „	0·6 g. av.	81	92	91	91	96	Slightly active
16	Green turnip shoots	0·7 g.	59	65	75	81	97	Active
17	„ carrot „	0·7 g.	67	70	70	—	—	Inactive
18	„ „ tops	0·6 g.	135	144	150	—	—	Active
19	„ maize (white) shoots	1·0 g.	61	65	70	75	83	Active
20	„ pea	2 shoots	79	90	102	113	119	Active
21	*Tradescantia* in soil	1·5 g.	101	104	112	112	—	Active
22	„ water culture	1·5 g.	105	116	123	—	—	Active
23	„ etiolated	1·5 g.	65	72	75	—	—	Active
24	„ iron free culture	1·5 g.	88	92	100	103	113	Active
25	Green cabbage leaves	1·5 g.	56	61	66	71	80	Active
26	White „ „	1·5 g.	56	53	55	57	60	Inactive
27	Mushrooms	1·5 g.	65	68	74	77	78	Very slightly active
28	*Ulva*	1·5 g.	91	106	106	108	110	Active
29	*Cladophora*	1·0 g.	100	105	118	123	137	Active
30	*Polysiphonia*	1·0 g.	82	92	91	97	90	Slightly active
31	*Chondrus crispus*	0·5 g.	120	125	129	128	—	Inactive
32	Protein ext. spinach	0·1 g.	82	89	95	100	104	Active
33	„ white cabbage	0·5 g.	95	90	91	91	—	Inactive
34	Unsap. matter from dried peas	0·02 g.	62	80	78	81	93	Active

[1] Weights during preliminary periods on basal diet are omitted for simplicity.

SUMMARY.

1. Dried seeds vary in their content of vitamin A, but are in general deficient in this substance.

2. The amount does not appear to be increased by germination.

3. Etiolated seedlings and leaves deficient in chlorophyll (white cabbage) do not apparently synthesise the vitamin.

4. Green leaves form large amounts of vitamin A, and from evidence obtained from water-culture experiments this synthesis may be effected from inorganic salts.

5. Lower plants (marine algae) containing chlorophyll synthesise this dietary factor; others which are differently adapted for photosynthesis (red weeds) are not so active in this respect, whilst those devoid of pigments which play a role in carbon assimilation (mushroom) are almost completely deficient.

6. The vitamin A in green leaves does not appear to be associated with proteins. It may be extracted in the fat removed by solvents and appears in that fraction of the fat which is resistant to saponification.

The thanks of the authors are tendered to the Medical Research Council who defrayed the cost of this investigation.

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LXVII. RESEARCHES ON VITAMIN A. VII. NOTES ON THE FACTORS INFLUENCING THE VALUE OF MILK AND BUTTER AS SOURCES OF VITAMIN A.

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During the last few years we have had occasion to examine a large number of samples of butter and milk for the presence of vitamin A, and from the examination of this material, drawn from many sources and representing a wide variety of products, we have obtained results which throw some light on the factors governing the distribution of the vitamin in these important foodstuffs. At the same time we have made a number of controlled experiments to gain more definite information on one or two points which have arisen during the course of the work. In the present paper we have put together what we regard as the most important results we have obtained, in spite of the fact that the whole does not form a connected survey.

Quantitative method of testing milk and butter for vitamin A.

The essential details of the method we have employed throughout this work have been described in a previous paper [Drummond and Coward, 1920, 1]. The procedure of giving the foodstuff to be tested as a small weighed or measured supplement to the experimental animals before they receive their daily ration of the basal diet is the only means of ensuring a quantitative intake, and should always be employed. By using this technique and by care in the selection of the test animals results may be obtained which are sufficiently accurate for the approximate estimation of the relative values of samples of milks or butters.

The most serious error in these experiments appears to be that introduced by the individual variation in the response of the test animals. This source of inaccuracy can never be entirely eliminated but it may be greatly reduced by care in their selection. In the routine testing of milks and butters it is our usual procedure to make the preliminary test by giving a group of animals whose growth has been satisfactorily inhibited by a diet deficient in vitamin A

a daily supplement of 2 cc. or 0·2 g. respectively. There is seldom if ever any difficulty in making the animals eat the rations and the method is not nearly so troublesome as might at first appear. As soon as the day's supplement is consumed the animals are given their ration of the basal artificial food mixture. From the results of the preliminary test an indication of the subsequent procedure may be obtained. If good resumption of growth is obtained with 2 cc. of milk or 0·2 g. of butter, another group is tested with amounts of 1 cc. or 0·1 g. respectively, whilst if no growth is obtained on the first trial larger supplements are given until positive results are obtained. In this manner a series of curves showing recovery of growth at various rates may be obtained, and from a comparison of these rates a rough idea may be gained of the relative value of the foodstuffs being tested.

We would like to emphasise, as previous authors have done [*e.g.* Chick and Hume, 1919], the importance of knowing the actual intake of the foodstuffs being tested. The procedure followed by some investigators of incorporating the substance with the basal diet and of making no record of food consumption cannot yield results which are in any sense quantitative.

The vitamin content of milk.

(*a*) *The influence of the diet of the mother.* It was originally shown by McCollum, Simmonds and Pitz [1916] and confirmed by Drummond [1918] that the milk secreted by the lactating female will tend to be deficient in vitamins unless her diet contains adequate amounts of these factors. More recently a great deal of experimental evidence has been obtained in support of this observation, and from our own observations we are inclined to believe that this cause is by far the most important in causing such variations.

Further reference to the influence of the diet on the food value of the milk, as illustrated by studies on vitamin A, will be made in the section on butter.

(*b*) *Influence of breed.* Our examination of milks has included a number of samples from cows of different breeds being fed on the same ration. These animals were all out on pasture which was at that time fresh and green. Unfortunately our results are not numerous enough nor do our observations extend over a period sufficiently long to enable us to arrive at a definite conclusion on the effect of breed on the nutritive value of the milk. The impression we gained, however, was that the milk obtained from cows of the Jersey and closely related breeds tended to be richer in vitamin A than that yielded by Shorthorns and Black Angus.

Much more work of a systematic nature is required to settle this point. It is interesting to recall in this connection the experiments of Palmer and his colleagues [1914] on the pigments of milk and butter. They showed that the yellow lipochrome pigments which naturally colour these foodstuffs are derived from the green fodder of the cow, and that they may be stored up in the body fat. The body fat of the Jersey breeds is much more deeply

pigmented than that of most other types and they tend to yield a milk fat of a rich golden yellow colour. Now it has been shown that the storage of such yellow pigments in the body fat may in some species be accompanied by a storage of the vitamin A when the animals are on pasture [Rosenheim and Drummond, 1920; Drummond and Coward, 1920, 2], and it is therefore conceivable that the cows of the Jersey breeds have a higher storage capacity for vitamin A than others such as the Shorthorns, as they undoubtedly have a higher capacity for storing pigments. This might perhaps explain the higher vitamin A value of the milk yielded by grass-fed Jersey cattle, or it may on the other hand be connected with the high fat content characteristic of those milks.

(c) *Seasonal variation.* As far as we can ascertain there is no appreciable variation in the vitamin A content of milk at different seasons of the year, apart from that associated with the different character of the diet ordinarily consumed at those seasons. The amount of our information on this point is however very scanty.

The physiological significance of colostrum.

The theories to be found in the text-books of physiology at the present time regarding the function of colostrum scarcely appear to be adequate to explain why so highly specific a secretion should be provided during the first few days of the life of the offspring. It may be true that this product has a laxative action, to quote one of the existing theories, but one instinctively looks for a more fundamental function for this characteristic fluid. Whilst we do not claim to have discovered this function, we would like to point out that under normal conditions colostrum appears to be considerably richer in vitamin A than the later milk. Illustrations of this may be seen from the curves in Fig. 1 which compare the growth-stimulating power of the milks yielded by the same animals.

We have obtained this type of result on several occasions with various samples of colostrum and later milk, but with one lot of samples little appreciable difference could be detected.

The significance of our observation may be slight, but on the other hand it may indicate that the child during the first few days of life requires a relatively high concentration of certain substances, amongst them the vitamin A, either for its immediate requirements or to provide it with a good reserve at the outset. We are inclined to regard this higher value of colostrum as an indication of a mobilisation of the reserves of the mother, since it does not appear to be proportional to the fat content. The results show that the concentration of vitamin A in the fat of colostrum is decidedly higher than in the fat of the later milk.

It is again interesting to recall that there is also a partial mobilisation of the lipochrome pigments of the mother's body fat for the production of

colostrum, which normally contains a much higher concentration of these colouring substances than the fat of the later milk.

At first our attention was directed to the high protein content of colostrum, since we were at that time considering the possibility of the vitamin A being of a lipoid character and attached to protein. We could not, however, obtain

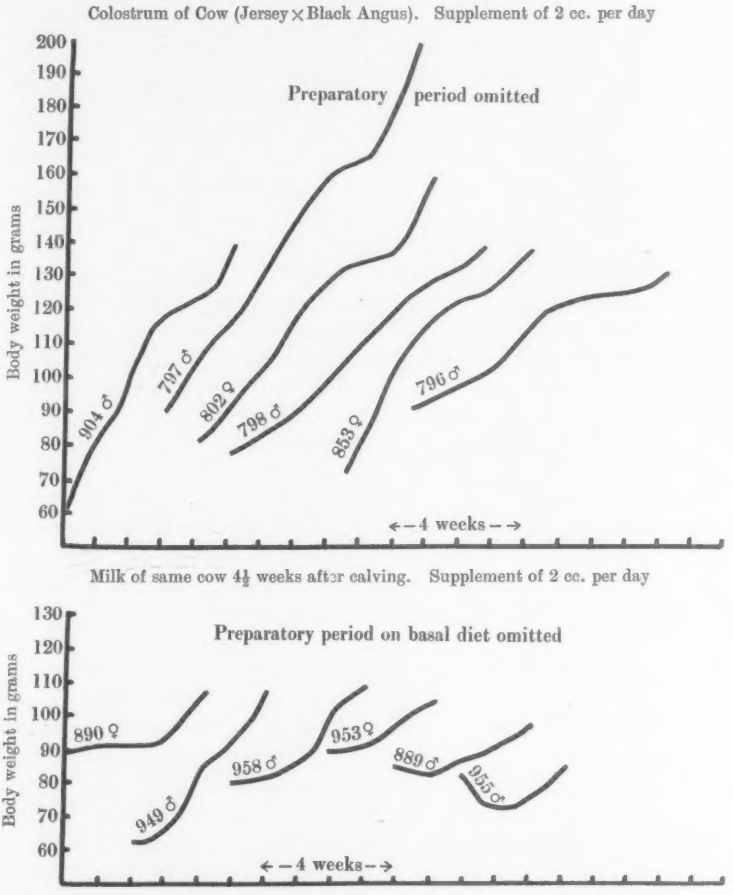

Fig. 1. Curves showing superior growth-promoting power of 2 cc. of colostrum per day to that of 2 cc. of later milk (lower curves).

any satisfactory evidence of the existence of such a protein-vitamin complex, and could not connect the high vitamin A value of colostrum with its high protein (other than caseinogen) content. From one experiment of somewhat short duration we learnt that there appears to be a gradual fall in the amount

of vitamin A in the secretion as the colostrum is gradually replaced by the normal milk.

These observations may be of significance in practical nutrition, and should be considered in relation to the giving of colostrum to infants.

The vitamin A content of butter.

(*a*) *The influence of butter-making.* Early in our investigations we were struck by the superior value of milk as a source of vitamin A over that possessed by the same quantity of milk fat in the form of butter. At first we were inclined to consider this in the light of the theory then occupying our attention that the vitamin exists in milk in the form of a complex with protein. The experimental facts have however led us to abandon this view and there appears little doubt that the loss of vitamin A in butter-making is partly mechanical and partly due to destruction.

As an example of the type of difference may be given the average figures we have obtained during our study of milks and butters. Fresh milk from cows fed on green pasture will in nearly all cases induce a resumption of growth in rats fed on a diet deficient in vitamin A when given in a daily supplement of from 2–3 cc. [cf. Hopkins, 1912 and 1920, 1]. This amount of milk may be regarded as supplying approximately 0·1 g. of fat per day. In order to induce the same amount of growth by adding a supplement of butter it is necessary to give about 0·2–0·3 g. per day.

In order to establish more definitely this relationship of milk and butter, a butter-making experiment was carried out through the kindness of Capt. J. Golding, D.S.O., at the Dairy of University College, Reading.

On June 2nd the morning milk of four shorthorn cows (first or second calf) was collected (7 gallons) and a sample of half a gallon immediately bottled and refrigerated for testing. The remainder was taken down to the college dairy, where, four hours after milking, it was separated in a centrifugal separator after pre-heating to 90° F. Four and a half pints of cream were obtained, and both cream and separated milk were cooled to 65° F. and placed in a cold store. Twenty-four hours later the cream was made into butter without having been submitted to any pasteurisation or artificial ripening process. The four and a half pints of cream were mixed with four and a half pints of water at 66° F., the mixture being at a temperature of about 52° F., and was churned in a 12-gallon churn of the ordinary type. Churning occupied half an hour and three pints of breaking water and a pint of rinsing water were used. Five quarts of buttermilk were obtained of which a sample was removed and refrigerated for testing. Two and a half gallons of water at 46° F. were added for washing, and, after draining through muslin, the butter was returned to the churn where it was salted by being turned a few times with 1½ gallons of water containing 3 lbs. of salt. After draining the butter was worked and made up into a block. The four products, fresh milk,

separated milk, buttermilk and butter, were kept in cold storage until used for testing on the rats.

The quantities used for testing were, original milk 2 cc., separated milk 2 cc., buttermilk 2 cc., butter 0·06 (the original milk contained 3·05 % fat) per day respectively.

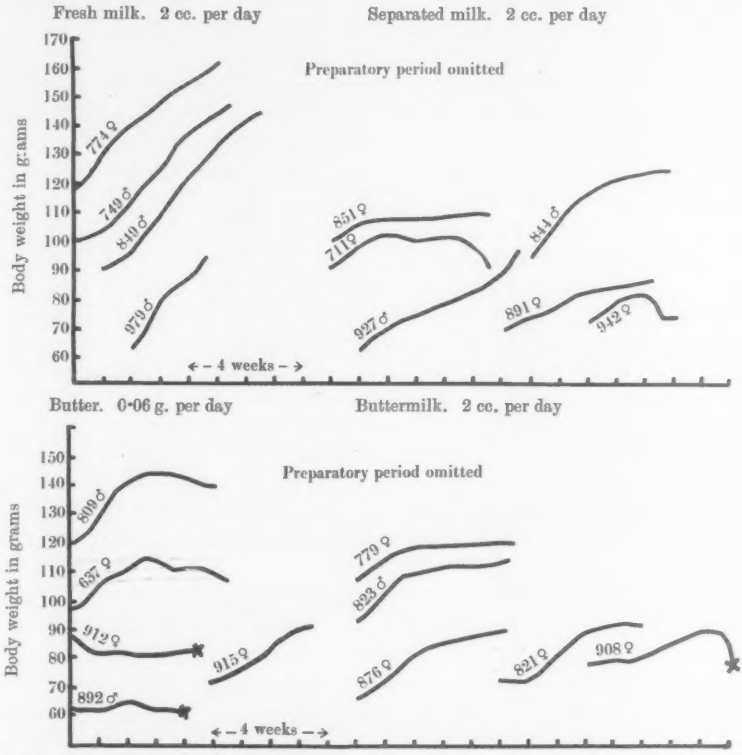

Fig. 2. Curves 774, 749, 849, and 979 show effect of 2 cc. original milk per day. The lower potency of the separated milk is shown in curves 851, 711, 927, 844, 891 and 942 and that of the buttermilk in 779, 823, 876, 821 and 908. The amount of butter equivalent to the whole milk gave much less growth (Curves 809, 637, 912, 915, 892).

As may be seen from the curves in Fig. 2 the rats fed on the supplement of 2 cc. per day of fresh milk resumed and maintained almost normal growth throughout the duration of the experiment. The rats to which approximately the corresponding amount of milk fat in the form of butter was given showed some growth (or none) for two weeks, when they ceased growth and declined. The rats fed on the supplement of separated milk (0·07 % fat) showed some growth, but in no case was it nearly so good as that given by the same amount of fresh milk, and later the growth curves became horizontal or in some cases declined.

The rats fed on the supplement of buttermilk showed growth very similar to that on the separated milk.

This controlled experiment confirms the opinion we had formed from the examination of a large number of miscellaneous samples that butter is usually a less potent source of vitamin A than the amount of milk which would yield that butter. The loss may apparently occur in more than one way. There is undoubtedly some loss by way of the separated milk which carries a small amount of fat containing vitamin A, but the possibility of loss occurring by chemical processes during the butter-making must also be considered.

It is difficult to estimate the chances of destruction during these processes, and although we know that the vitamin is readily inactivated by contact with air or oxygen, especially at high temperatures [Hopkins, 1920, 2; Drummond and Coward, 1920, 3; Zilva, 1920], the likelihood of such destruction having occurred in our experiment to any marked extent does not appear to be great. Possibly the large surface of the fat exposed to contact with air during churning may lead to some inactivation, since we know from the observations of Rogers [1909] that the amount of air entrapped in the butter during working, especially overworking, may cause oxidation of the non-fatty constituents. Although Hunziker [1920] does not entirely agree with the conclusions of Rogers he states that it is unquestionably true that any air permanently present in the butter becomes very finely divided and emulsified by overworking, and in this form a much larger surface area of the butter becomes exposed to the oxidative action of the air. Destruction of the vitamin by oxidative processes from this cause more probably occurs slowly during storage than in the short time occupied by the actual butter-making.

In much of the making of butter the cream undergoes several important processes which were not employed for the sample investigated above. The chief of these are pasteurisation and ripening, but the extent of the destruction during these processes cannot be judged until controlled experiments have been made with the various types of process commonly in use, and statements such as that of Hunziker [1920, p. 572] cannot be accepted until such experiments have been carried out. In this connection we may say that in examining average samples of London milk, all of which have been pasteurised by one process or another, we have encountered samples with a fairly high vitamin value, a finding that may perhaps indicate that in ordinary circumstances the loss by pasteurisation in milk at any rate is probably not serious. Daniels and Loughlin [1920] in a recent study of milks were unable to detect any loss of vitamin A by heat treatment, but a study of cream has not so far as we are aware been made. With regard to the effect of the ripening of cream for butter-making on the amount of vitamin present we have also no definite evidence. The process entails little contact with air and the temperature is kept low so that it is likely that little or no destruction of the factor occurs. On the other hand there is no justification for assuming as some have done

that the growth of the organisms will increase the amount of vitamin, since we have evidence that this substance is not produced by the lower plants in the absence of chlorophyll [Coward and Drummond, 1921].

Reference to any standard work on the butter industry such as the valuable book by Hunziker [1920] shows that in some cases the cream is subjected to processes which must be suspected of causing more damage to the vitamin A than those we have already considered. These include the aeration or blowing of cream, a procedure which is believed by some to be of value in removing objectionable odours. Thus, for example, Ayres and Johnson [1914] demonstrated that certain undesirable flavours may be removed from cream and milk by blowing with a vigorous current of air at a temperature of 145–160° F. for a period of five minutes or more. The existing knowledge is sufficient to justify the conclusion that such a process would very materially lower the amount of vitamin A present.

In some cases considerable use is made of the process of neutralising acid cream to render it fit for butter-making.

It appears probable as Hunziker points out that the process of neutralisation of cream will cause little if any loss of vitamin A.

(*b*) *Butter as a source of vitamin A.* The early work in the study of the vitamins led to the belief that butter is a very rich source of vitamin A, and in a recent compilation of the knowledge of these substances [Medical Research Council, 1919] it was placed high in the list of foodstuffs. Since that list was compiled considerable advances have been made, and we now appreciate the very great importance of two facts, namely, that samples of butter may show a wide variation in the amount of vitamin present, and that in comparison with some other fats butter is not to be regarded as possessing so high a potency as was at first believed. Appreciation of these two points may help to explain certain of the discrepancies which have been encountered by some workers, particularly by those who are studying the vitamins in their relation to practical infant feeding.

The use of the roughly quantitative method of testing which we have elaborated enables us to form an approximate estimate of the relative value of individual samples of butter, and some which we have investigated have possessed a growth-promoting power little or no better than the average refined vegetable oils, which are usually classified low down in the list of foodstuffs containing vitamin A. Such samples are in nearly every case derived from cows on dry feed during the cold weather or when fresh green pasture is not available. Further confirmation of the influence of the feed on the vitamin value of the butter which was pointed out in a previous communication by Drummond and Coward [1920, 2] is afforded by the curves in Fig. 3. These curves represent the testing of samples of butter from a farm herd of Shorthorns. The first sample was obtained at the end of April 1921 and was typical of the butter produced by the cows then fed in stall on hay, cake and roots, being a hard, friable and almost colourless product. As

may be seen from the curves in Fig. 3 this sample when tested in 0·2 g. per day had practically no value as a source of the vitamin.

A sample of the butter from the mixed milk of the same herd was obtained after they had been put out to grass for one week, the grass being at that time fresh and green. This sample was more highly pigmented with lipochromes—none of the samples contained added pigment—and was distinctly softer than the first sample. The effect of the short period on grass in raising the amount of vitamin present can be seen from the curves in Fig. 3 and is also seen in the case of a third sample prepared after the animals had been out for nearly a month. As is well known this year has been remarkable for a most prolonged and severe drought which has gradually dried up and withered the pasture land to a degree seldom seen in this country.

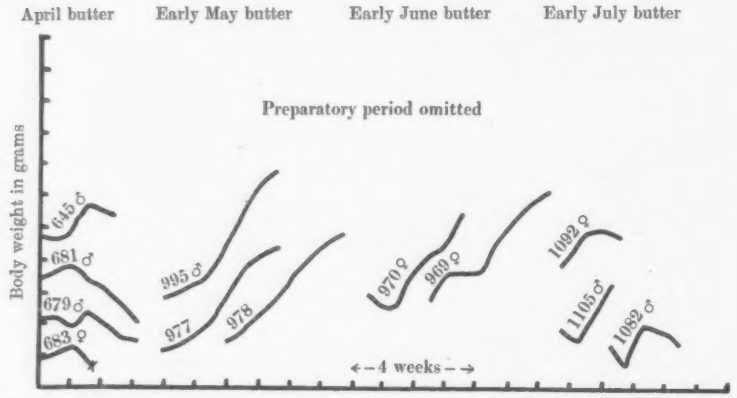

Fig. 3. Effect of diet on vitamin A value of butter. Curves 645, 681, 679, and 683 represent 0·2 gm. daily of butter from winter fed cows in April. The cows were put out to grass and the food value of the butter immediately rose as seen in the tests of 0·2 g. of the early May butter (curves 995, 977 and 978) and the early June butter (curves 970 and 969). The severe drought has caused a fall in the amount of vitamin present in July butter (curves 1092, 1105, 1082).

The farm in Buckinghamshire from which the samples of butter were obtained suffered very badly from the lack of rain so that before the beginning of July the pasturage was insufficient to support the cows, and it was necessary to fall back on cake. The effect of the drought in drying up the fresh pasture and of the cake feeding is already apparent in the marked falling off of the food value of the butter.

Further experimental confirmation of the relation of the food of the cow to the vitamin content of the milk has recently been brought forward in a preliminary communication by Hughes, Fitch and Cave [1921].

The storage and preservation of butter. We have investigated a number of samples of butter which have been in storage for various lengths of time,

usually at low temperatures, and have not found them to possess a general lower average value than fresh samples. No sample which we investigated had, however, been stored for a longer period than eight months.

In these samples the value as a source of vitamin A appeared to be very much more dependent on the season at which it was placed in store, *i.e.* the diet of the animals at that season, than on the length of time it had been in storage. We have encountered one sample of Danish tinned butter which had been in tins for at least six months but which had a very high potency when tested for the vitamin. This sample had been derived from grass-fed cattle and was deeply tinted with the natural pigments derived from the green fodder. Our experience indicates that butter stored at low temperatures (below 10° C.) will not suffer any appreciable loss of vitamin if undue exposure of surface to air is prevented and the other conditions of storage are good[1].

We have also examined samples of beef fat (oleo-oil) from S. American grass fed cattle, and have found them to show as high a potency after many months' storage in tins as is possessed by good samples of butter fat.

If, on the other hand, the conditions of storage are such as to permit definite even if slow oxidation processes there will certainly be danger of loss of vitamin. This type of change is apparently very much more likely to occur in butters which have been made from "high acid" creams or which have been overworked [Hunziker]. The oxidation which occurs in such butters is according to Dyer [1916] that of the non-fatty constituents of the buttermilk, but we have encountered many cases where a marked loss of natural pigment due to oxidative bleaching of the lipochromes has occurred in such butters.

The loss of the natural pigments, carotene and xanthophyll, in butters on storage is in all the cases we have examined accompanied by a loss of vitamin, but this does not frequently occur unless the fats are exposed to light and warmth as well as air. We have tested one sample of the so-called "tallowy" butter produced by the prolonged exposure to air and light at room temperature, in which the oxidative processes had bleached all trace of pigment substances and given rise to an objectionable tallowy odour, and found that there had also been a complete destruction of vitamin A.

It is today well recognised that sweet cream butter stored at low temperature will not develop what is termed the "storage flavour" so readily as that prepared from pasteurised or neutralised cream. This development of "storage flavour" appears to represent a very early stage in the oxidation processes which lead on to the appearance of more objectionable odours, and eventually to rancidity [Rogers, 1913]. We have not noted any appreciable change in the vitamin value of butters showing the "storage flavour," but this may be due to the fact that our test method is not accurate enough to detect the slight change which might have occurred in the short time during which the changes had proceeded.

[1] This is in agreement with the results of Harden and Zilva (private communication) who found no detectable deterioration in the value of butter kept in cold storage for twelve months.

Rancidity. The groups of processes which together produce rancidity are very incompletely understood, but we know that the two most important types of reactions occurring, in most cases simultaneously, are, a hydrolysis of the glycerides and obscure changes of an oxidative character. When butter is so exposed that the second type of reaction is especially well favoured, we have found that rancidity is accompanied by a comparatively rapid loss of vitamin A. This is particularly marked if a large surface of the fat is exposed to air at moderate temperatures. On the other hand we have noted samples of butter protected from the light in which a certain degree of rancidity, as measured by the acid value, has developed without marked loss of vitamin. It would therefore appear that destruction of the accessory factor during rancidity will depend on the extent to which oxygen plays a part in the process.

In this connection we may mention the unpublished results obtained by Mr S. N. Ghose working in this laboratory, who has found that average samples of unadulterated Indian cow's ghee have approximately the same value as average samples of mixed butters. In the preparation of this product the freshly boiled milk is inoculated with sour milk and allowed to curdle. It is then churned and the fat is allowed to become rancid after which it is clarified. Whilst the rancidity develops the fat is kept out of contact with air and away from the light and heat.

Renovated butter. The practice of renovating butter which has become rancid and thereby reconverting it into an edible product appears to have died out in Europe. At any rate we were unable to ascertain where such products are generally made or to obtain more than one commercial sample. Apparently the manufacture of this product still exists to a considerable extent in America, most of our information having been derived from that source. According to Hunziker the usual American process involves prolonged aeration in large jacketed cylindrical tanks by means of a powerful current of air blown through a distributing rosette near the bottom until it has become "neutral," that is until the objectionable odours have disappeared together with most of the volatile fatty acids.

It appears to us highly improbable that any appreciable amount of vitamin present in the original butter could survive so drastic a process of purification. As we have remarked before, the employment of a soured milk to restore or develop the butter flavour would not add, in our opinion, any amount of vitamin A to the final product, unless where, as appears to happen in a few cases, whole milk is used.

Dr L. H. Lampitt has very kindly furnished us with information of the processes usually employed when butter was renovated in this country, and we have followed out the influence of one of these methods on the vitamin present in a sample of butter which had been fully tested in the fresh condition.

The sample of butter chosen was a good specimen of Danish dairy butter from cows out on green pasture, 0·2 g. per day of which supplied the requirements of the experimental animals for growth. A sample of this butter was

allowed to become rancid by exposures to slight warmth and light for two weeks, after which it was again tested for activity. Its value was found to be very considerably lowered, 0·4 g. giving fairly satisfactory growth.

The rancid sample was then "renovated" by the following method. The fat was melted at low temperature and allowed to separate from the aqueous layer which was drawn off carefully. The liquid fat was then subjected to the passage of a current of live steam for half an hour to remove volatile components of an unpalatable nature. The deodorised fat was then shaken up vigorously with an equal volume of slightly soured separated milk, and the regenerated butter tested on rats in the usual manner. The results of the test show that little further loss of vitamin A had occurred as a consequence of the renovation.

A commercial sample of renovated butter fat which had not been reincorporated with milk was found to possess a high value as a source of vitamin A. No information of the method used in its preparation could be obtained. It possessed a marked tallowy odour and was most unpalatable, but the natural pigments of the butter had not been altogether destroyed.

Summary.

1. The diet of the cow is undoubtedly the chief cause of variations in the amount of vitamin A in milk. Season, apart from the consequent diet, and breed are not the cause of any marked variation.

2. Colostrum normally proves to be richer in the vitamin than the later milk.

3. Butter appears to be less potent as a source of fat-soluble A than the same amount of fat supplied in the original milk. The loss as far as we can ascertain is partly mechanical and partly due to destruction.

4. Certain processes used in butter-making which involve exposure to air at high temperatures are believed to cause a loss of the A factor. This applies especially to some of the less frequently adopted methods such as "blowing" to remove objectionable odours.

5. Butter shows wide variations as a source of vitamin A. Most of this variation arises from differences in the diet of the cow. We have confirmed our previous observations on the low vitamin value of butter produced during the winter months from stall-fed cattle on dry feeds of hay, roots and cake. Even the drying up of pasturage in a hot summer may lower the value of the butter.

6. The storage of butter and its preservation by such methods as tinning do not appear to lower the vitamin A value unless changes of an oxidative character take place. Modern improvements in methods all tend to minimise loss from this cause.

7. Destruction of vitamin A occurs during the processes of rancidity if oxidation plays much part in this change, but considerable development of

free acid may occur without loss of the accessory factor if oxidation—as indicated by a bleaching of the natural pigment—is prevented.

8. The "renovation" of rancid butter will entail further loss of vitamin if the methods employed are likely to cause oxidation.

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LXVIII. ON THE THEORY OF GELS. III.

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In earlier papers [1918; 1920, 1 and 2] much evidence has been adduced in support of the hypothesis that the reversible gelation of the natural emulsoids is an extreme case of crystallisation and takes place in conformity with von Weimarn's formula $N = K.\frac{P}{L}$. The very small diffusion constants of the gel substances greatly retard the velocity of crystallisation and determine a large degree of supersaturation, P, in the cooling sols. In this stage the liquid contains large numbers of aggregated particles and is highly viscous. The factor K is, therefore, large. And, since the solubility, L, is small, each factor contributes to a maximum value of N, which corresponds to the formation of ultramicroscopic particles.

The very small rates of diffusion of the natural emulsoids have been attributed, partly, to high molecular weight, and, partly, to a chemical, or other, tendency to molecular aggregation. The colloidal effect of both these causes is exhibited by a great many organic substances, of which examples have been quoted. And, indeed, it has been estimated recently [Lloyd, 1920], that gelatin may have a molecular weight of 10,300, which, alone, would be sufficient to confer upon this substance its colloidal properties. A review of all the phenomena of gelation not only shows them to be compatible with the process of crystallisation, but strongly suggests that the two processes are the same. In two cases, viz., the thermal anomaly and heat of swelling of gelatin, where the recorded properties did not agree with the requirements of the theory, the experimental data were shown to be in error. Moreover, von Weimarn's prediction that colloids have a solid crystalline phase has been verified in certain cases by X-ray analysis [Debye and Scherrer, 1916; Scherrer, 1918].

The question may be asked, however, whether gelatin has a definite solubility which may be used in von Weimarn's formula. An affirmative answer is supplied both by the molecular theory of solution [Bradford, 1919] and by experiment. The electrical structure of matter indicates that all molecules attract one another, whether alike or unlike. And solution is shown to be due to the disintegration of the solid, by the kinetic energy of its particles, when its surface tension has been reduced by the counter-attraction of the solvent in which it is immersed. Aided by this counteracting force, particles at the surface of the solid that acquire a velocity, normal to the surface, above a certain limiting value, will be able to overcome the attraction of

adjacent solid particles and pass into the solvent. On the other hand, those particles of the solute that approach the surface of the solid from solution will be reclaimed, on account of the greater resultant attraction of the solid. Equilibrium will be attained when the numbers of particles entering and leaving the solution are equal. This condition determines the solubility of the solid.

If the ultimate particles of the solute attract one another more than do those of the solvent, usually the adhesion of the unlike particles will be intermediate between these two values. It follows that solutes with a greater molecular cohesion than that of the solvent will be the less soluble the greater their cohesion. The adhesion of solute and solvent is reflected in the changed surface tension of the latter. It is important to notice that the less soluble a substance, the greater its attraction for the solvent. If, as seems probable, these principles apply to colloid solutions, the "lyophile" substances have comparatively little attraction for their dispersion media, and conversely.

Since the kinetic energy of the solute particles varies with the temperature, the equilibrium conditions corresponding to saturation will also change. But, if the excess of solid be removed, entirely new conditions will prevail. A statistical equilibrium between the number of particles entering and leaving the solution will be possible no longer, until, with falling temperature, the solid has been re-formed. This can result, only from the aggregation of the solute particles in consequence of their mutual resultant attraction[1]. When two particles of the solute come into collision, aggregation will occur only when their relative kinetic energy is less than a certain limiting value that is sufficient to overcome their mutual attraction. On the other hand, when the internal energy of the associated particles reaches this limiting value, the union will be severed and the particles will be free to pursue their separate paths. Until the stage is reached, when the aggregates become large enough to subside, the problem is similar, analytically, to that of the dissociation of a gas, such as iodine vapour, investigated by Boltzmann [1898]. Analysis shows that the number of re-associated particles increases exponentially as the temperature decreases. Since larger particles may be formed by union of smaller ones, the particles will grow in size with falling temperature until they become big enough to settle. This stage will be reached when the force of gravity on the particles becomes sufficient to dominate the kinetic energy that tends to re-distribute them through the solution. Perrin's calculations indicate that, if n_1 and n_2 are the numbers of particles of radius r, at the heights x_1 and x_2, the distribution of the particles is given by the equation

$$\frac{n_2}{n_1} = e^{-\frac{N}{RT}\cdot\frac{4}{3}\pi r^3(\rho_1-\rho_2)(x_2-x_1)g}$$

where ρ_1 and ρ_2 are the densities of the particles and of the medium respectively.

[1] There will be, always, a resultant attraction between the particles of the solute whether their cohesion be greater, or less than, that of the solvent.

This gives

$$r = \sqrt[3]{\frac{RT}{N} \cdot \frac{\log \frac{n_1}{n_2}}{\frac{4}{3}\pi g(\rho_1 - \rho_2)(x_2 - x_1)}}.$$

The condition that the particles shall separate is that $\frac{n_2}{n_1}$ shall be small. If we suppose $\frac{n_2}{n_1} = \frac{1}{100}$ when $x_2 - x_1 = 0{\cdot}1$ cm., and give ρ_1 the value 1·16 which has been adopted below for the density of the gelatin particles, we find

$$r = 0{\cdot}06\ \mu.$$

When particles of the supersaturated solution reach such a size, they will begin to subside. The number of particles which separate will increase rapidly as the temperature falls, and, on these, the excess of solid will be deposited, until, once more, the statistical equilibrium between the solid and its solution has been re-established. Evidently the metastable limit is determined by the rate of aggregation of the solute particles. And, as von Weimarn has already indicated, even a metastable solution will deposit solid in course of time.

From this point of view, every substance must have a definite solubility, however small, in a given solvent, with which it does not combine chemically, without regard to the size of the particles which go into solution; except that the solubility will vary with the dimensions of the particles, in so far as these affect the internal field exerted by them. If, therefore, it be permissible to use the term crystallisation in the most general sense, to imply merely the separation of solid from solution, without necessarily imputing a definite orientation to the particles of the solid aggregates, although there is no definite evidence that any substance is truly amorphous, it may be admitted that there is good reason for regarding gelation as a crystallisation process and applying von Weimarn's formula to it.

The experiments, about to be described, show that gelatin conforms in every way to the principles laid down. Particles of macroscopic size have been obtained by decrease in supersaturation, P. But since the extreme values of K and L remain, the particles occur in the form of spherites rather than of regular crystals.

In the first experiments 0·3 and 0·5 % sols of Nelson's "crystal" leaf gelatin were allowed to evaporate spontaneously in shallow dishes at room temperature with addition of various antiseptics. The clear liquids developed, within a few days, an extraordinarily dense opalescence, which gradually disappeared as a light buff-coloured precipitate was deposited. This consisted of spherites up to 2·8 μ. Similar results were obtained by experiments carried out at 31° and 40°. The appearance of an opalescence indicates that gelatin is not completely miscible with water even at the higher temperature.

With the object of avoiding the addition of antiseptics, the experiments were continued by sealing slightly supersaturated solutions in tubes and allowing them to stand at room temperature. In the first place, 0·4 % gelatin solutions were filtered into chemically clean test tubes, quickly heated to

boiling and the ends of the tubes drawn out and sealed. The characteristic opalescence soon made its appearance, though more slowly than when the liquid could evaporate spontaneously. But, after a time, the remarkable fact was observed that the opalescence was slowly subsiding in the tube. The average rate of fall was found to be 0·10 during the first 20 days. A second measurement, five days later, gave a velocity of 0·18, and at the same time the milkiness had considerably increased. Several later observations showed a lower rate of fall about 0·07. Corresponding to the decrease in the velocity of sedimentation, the edge of the surface of the cloudiness appeared to be separating from the side of the tube, and the precipitate had the appearance of having coalesced to a just coherent jelly. On inverting one of the tubes the jelly broke up into flocks. But the unbroken gels continued to shrink and eventually came away from the sides of the tubes.

The experiments were continued with solutions of ashless gelatin made from a sample kindly supplied by the Osmosis Co., of which the moisture content was 14·74 % and the ash only 0·02 %. A 1 % sol of this gelatin was prepared by making up 1 g. of material to 100 cc. with water in a stoppered test mixer, allowing to soak over night and quickly warming until melted, by careful immersion in the water-bath. The warm liquid was filtered through Chardin's filter paper into a burette, the first portion rejected, the temperature noted, and small measured volumes run into a number of chemically clean test-tubes. The temperature of the liquid remaining in the burette was again noted and, from the average value, the true volumes at 15° of the gelatin sol used could be calculated: with an average temperature of about 35°, the correction was negligible. The necessary amount of water was then added to the test-tubes so as to make the gelatin contents range from, say, 0·25 to 1·0 % of dry gelatin.

The liquid in the tubes was quickly boiled and the tubes sealed. The actual strengths of the sols, three of each, in a typical experiment, calculated to dried gelatin, were 0·26, 0·30, 0·41, 0·51, 0·77 and 1·02 % respectively. The more dilute sols were quite clear at first, but all became distinctly opalescent within 24 hours, according to concentration, the 0·51 % tubes being very opalescent. During the same time the 0·77 % sols had set to very opalescent and just coherent gels; while the last three tubes were found to contain a slightly stronger jelly that was also still rather more opalescent. In about three weeks a film of clear liquid had collected on the surface of each of these six gels.

The opalescence in the remaining tubes, containing the weaker sols, developed from day to day and, after about ten days, the commencement of the subsidence of the fine particles was observed. The rate of fall was measured from time to time, and the distances fallen plotted against the time. The curious fact was thus brought out that there appear to be three distinct stages in the fall of the surface of the opalescence: (*a*) a long gradual fall at a uniform rate for from about 20 to 40 days according to the concentration, (*b*) a sudden

sharp drop taking place within a short time and (*c*) a slight continuous shrinkage at a slower rate than (*a*) which was still going on after a couple of months from the time of the sudden coalescence. The photograph, Pl. III, *b–k*, shows the final appearance of two tubes of each concentration, except the highest, after nine weeks from the beginning.

If the volumes of the precipitates are plotted against the concentrations, the curve cuts the concentration axis at the point corresponding to 0·12 g. of gelatin per 100 cc. of solution. This is, evidently, the true solubility of gelatin at room temperature, which remained fairly uniform at 18° during this time. According to the theory discussed, a slightly higher concentration should yield a metastable solution, which would remain more or less permanently supersaturated on account of the low diffusion constant of gelatin. This was found to be the case. A 0·12 % solution continued perfectly clear with no trace of opalescence, while a 0·13 % solution developed, in a few days, a beautiful clear bluish opalescence quite different from the appearance of the more concentrated solutions when in course of precipitation. At the time of writing this opalescence has in some cases persisted for four months, Pl. III *a*. The preparation is evidently a stable aqueous sol of gelatin. With a 0·135 % solution the metastable limit was overstepped and a minimum of flocculent precipitate was deposited.

The long gradual subsidence (*a*) of the surface of the cloudiness appears to correspond to the free fall of the gelatin particles, and the application of Stoke's law should give some indication of their relative sizes. It is interesting to see how these compare with the excess concentrations, P, in the light of von Weimarn's formula. In the table below, the radius, r, of the particles has been calculated on the assumption that these have a density of 1·16[1].

Reference number	Concentration $\left(\frac{\text{grams}}{\text{100 cc. solution}}\right)$	P $(=C-L)$	$\frac{P}{L}$	Rate of fall (mm./day)	r (μ)	$r \times \frac{P}{L}$
10	0·26	0·14	1·17	0·35	0·10	0·12
11	0·26	0·14	1·17	0·30	0·10	0·11
13	0·30	0·18	1·5	0·17	0·07	0·11
14	0·30	0·18	1·5	0·14	0·06	0·10
16	0·41	0·29	2·4	0·09	0·05	0·12
18	0·41	0·29	2·4	0·08	0·05	0·12
19	0·51	0·39	3·3	0·06	0·04	0·14
21	0·51	0·39	3·3	0·05	0·04	0·14

As has been shown, von Weimarn's formula is still in an indefinite stage. The variable N is defined as the precipitate form-coefficient, so that, as N increases, the size of the ultimate particles diminishes, until, finally, ultramicroscopic dimensions are reached. But whether N represents, inversely, the volume, surface or radius of the particles is uncertain. The last column of the table seems to show that the product of the radius and P/L is constant,

[1] By supposing the particles to be half water, this value was derived from the density of the air-dry Nelson's gelatin, $D_4^{15} = 1{\cdot}3329$ *in vacuo*, determined by weighing in air and in a sp. gr. bottle containing toluene.

indicating that N is really the reciprocal of the radius of the particles. Some hundred experiments in all gave similar results. The investigation is to be continued, a thermostat being used, so that separate series can be run at different temperatures to collect material for the determination of the solubility curve of gelatin, which may throw more light on von Weimarn's formula.

The advantage of using gelatin, rather than a mixture of salts in a double decomposition, for the further investigation of this expression is that the undoubted influence of the varying concentrations of the several reaction components on the solubility and metastable limit of the precipitate is eliminated. Moreover, on account of the very low velocity of crystallisation of gelatin, the disturbing effect of the concentration of the substance to be precipitated on the velocity of the condensation is largely avoided. This concentration affects not only the rate of growth of the crystals after they are started, but also the rate of precipitation of the initial nuclei. With high concentrations the great velocity of precipitation may prevent the calculated value of the supersaturation being reached. Above a certain limit, precipitation may be practically instantaneous, and no further increase in concentration could increase the actual supersaturation. This may explain the constant dispersity of condensed water particles above a certain degree of supersaturation [Wilson, 1911, 1912].

The more or less sudden fall (b) of the gelatin particles corresponds with the formation of an apparently coherent jelly. It is not advisable, at present, to speculate on the exact causes of this coalescence, except to suggest that it may be due to an attractive force between the particles. This seems also to afford a possible explanation of the gradual shrinkage (c) of the jelly, that follows, as being due to a gradual rearrangement of the jumble of particles resulting from the sudden fall together. The hypothesis has the advantage of suggesting a physical cause for the syneresis, and seems preferable to the mere application of the term "dehydration." The mutual attraction of the particles may also be the origin of the opposing elastic force that must be assumed in cases of imbibition; as the attraction between the gel particles and those of the liquid, together with the capillary effects, is the origin of the swelling.

Microscopic examination of the precipitates in the tubes gave the appearance of particles just too small to be differentiated, like sand collected in ridges. There were also a few small round grains just large enough to be seen. Some of these were aggregated in clusters of two or three or more. With 0·2 % gelatin the ground-work of undifferentiable particles was marked, and the few larger grains reached nearly 1 μ in size. With increasing concentration the sand-like appearance diminished in intensity and the larger grains became smaller, more aggregated and more numerous. With 0·3 % gelatin the ground-work was still clearly visible, while there were a good many larger particles just differentiable, mostly aggregated in clusters. With 0·4 % the sand-like appearance was only just to be made out, and the larger granules were

numerous, only just distinguishable and often aggregated into large clusters or bundles of fibrils.

Thus the hypothesis that the natural emulsoid gels have a granular structure like that of a pile of shot receives further confirmation. The individual spherites would seem either to be aggregated crystallographically, or to adhere by their mutual attraction. It will be admitted, however, that, with such very small particles, there can be little difference between these two methods of attachment, since, in either case, the union would be due to the forces between the few molecules in the surface of contact. The coupling would be unstable in the case of only two grains, but would become firm as more grains were added.

To avoid the possibility of bacterial influence, Prof. Paine very kindly steamed some preparations three times on consecutive days after sealing. Although some of the gelatin was destroyed by the process the same series of effects was obtained. The 0·2 % solutions remained perfectly clear, the 0·35 % preparations produced opalescent metastable solutions, or sols, one of which persisted for some months. While the 0·85 % tubes developed a precipitate comparable in bulk with that in the 0·26 % unsteamed tubes.

Similar experiments were made with agar that had been dialysed in the stick form for ten days in the presence of a trace of camphor. These behaved in the same manner, but the opalescence was much weaker. A solution corresponding to 0·05 % of air dry agar became just opalescent and deposited a trace of precipitate in 14 days. This consisted mainly of spherites up to 2 μ. With 0·075 % agar the opalescence was still only just noticeable and a small quantity of flocculent precipitate collected at the base of the tubes. A 0·1 % solution gave a more distinct opalescence which gradually subsided and the opalescent portion eventually set to a jelly that occupied just two-thirds of the original volume after four months, Pl. III, *l*. With 0·125 % agar, the whole set to a just coherent gel that showed syneresis and contained some, more or less rounded, more cloudy portions. The 0·05 % jelly also showed syneresis, was opalescent and contained bands of more cloudy gel at 2·1, 2·35, 3·25 and 3·35 cm. below the surface. The 0·2 % jelly was more coherent and nearly homogeneous.

These experiments establish that gelatin, and presumably agar, behave to water exactly as do other substances in regard to suitable solvents. Gelatin has a definite small solubility at room temperature. At a slightly higher concentration it forms an ordinary metastable solution which tends to be permanent because of the very slow rate of diffusion of the substance. This solution is beautifully opalescent on account of the size of its solute aggregates and may be regarded as a typical sol. With further increase in concentration, a precipitate is produced, and the bulk of the precipitate increases with the concentration until, as in many other cases, it fills the solution. Concurrently with increasing concentration, the particles of the precipitate grow smaller, as required by von Weimarn's theory, until, when their distribution is sufficiently dense, they form an apparently homogeneous gel.

A further test of the theory is readily suggested. Substances which form gels only under suitable conditions revert, more or less rapidly, to the ordinary crystalline state; while the natural emulsoids appear to persist in the gel form. The difference must be due to the slow diffusion constants of the latter substances that retard their re-crystallisation. Therefore, if such gels be kept for a sufficient time, signs of re-crystallisation should be developed. Actually the presence of microscopic spherites in aged silicic acid gels has been described on several occasions. And some geological specimens of sponge spicules have a radiating definitely crystalline structure. Very likely this has developed in the course of ages from the opaline silica of which, probably, the spicules were originally composed.

It is easy to test the theory in the case of gelatin. A 5 % solution, made from Nelson's "crystal" leaf gelatin was filtered, boiled and sealed in tubes. The gels had the usual appearance, showing practically no opalescence and were perfectly clear. But, after keeping for three months, a distinct opalescence had developed, which grew from month to month, until at the time of writing, after ten months, it has become very marked and almost as deep as that of the 0·13 % metastable solutions of ashless gelatin. Microscopic examination of one of the gels after four months showed an almost clear field in which some tiny round particles were just to be seen. With lapse of time the visible particles gradually developed in size and number, and, now, numerous spherites may be observed from just distinguishable up to 1 μ. These must have grown from the larger amicroscopic grains of the gel at the expense of the still smaller ones, just as in the case of any ordinary substance in contact with a solvent.

Using the term in the general sense, the case for the crystallisation of gelatin appears to be fairly complete. But the exact nature of the ultimate particles of the gel remains to be determined. When large enough to be seen, gelatin, agar, silicic acid and starch particles are invariably spherical. Since the radiating structure is evidently that most readily assumed by crystallising substances, complex bodies may be expected to take this form if they crystallise at all, and this is found to be the case, generally, with complicated organic substances. But spherites are known in every gradation from the obviously crystalline form, built up of coarse radiating crystalline needles, separately visible, through stages showing only a more or less radiating formation, but which give the well-known shadow cross in polarised light, to apparently homogeneous spherical bodies giving no definite evidence of crystalline structure. This limiting stage may, or may not, be crystalline. In the case of gelatin, Scherrer's analysis does not show, necessarily, that the amicroscopic spherites are not crystalline. Their tiny radiating filaments would have a cross section of, perhaps, not more than two or three molecules, and could hardly be expected to give interference with monochromatic X-rays. Moreover, neither the complex structure of the molecule, nor the mixed chemical composition of the substance would favour a positive result.

Spherites of starch have been described by a number of observers. The writer obtained them, in a similar way to Bütschli, by the spontaneous evapo-

ration of carefully filtered, boiled aqueous solutions of starch. They showed no shadow cross [Meyer, 1914] and appeared to consist of aggregates of tiny "drops." But there is not much doubt that natural starch grains are actually crystalline, and Bütschli [1898] has prepared artificial grains exactly resembling them. It seems likely, therefore, that the tiny drops, or smallest particles, composing the globular aggregates of starch may be crystalline.

Probably the difficulty in preparing definitely crystalline grains of gelatin may be due to the fact that it is not a chemically pure substance. Since the spherites show much resemblance to the grains of mastic and gamboge prepared by Perrin, it seemed useful to try to grow some of these large enough to be examined in polarised light, though, again, neither of these substances is a definite body.

Prof. Perrin very kindly supplied the information that large grains, up to 10 μ, could be obtained by slow diffusion of the alcoholic solutions into water. The nose of a dropping funnel containing the clear filtered 4 % alcoholic solution was passed through a cork into a small 50 cc. cylinder full of water, and diffusion allowed to continue for about five weeks. The resulting grains were washed by decantation and examined with a $\frac{1}{15}$" objective.

Beautiful mastic particles were obtained of all sizes up to about 9·2 μ. The smaller particles, up to about 3 μ, appeared translucent, but the larger ones had a mosaic appearance, as if built up of aggregates of the very small particles that were also present. The latter showed fairly vigorous Brownian movement and appeared to be attracted by the larger spheres. But some cause, possibly the energy of their motion, seemed usually to prevent their coalescence, though occasionally a small particle might be seen to be captured.

The gamboge grains were slightly larger, up to 10 μ, and mostly quite clear, though some appeared mosaic. In this case there were very few of the smallest particles, and this suggests the possibility that the mosaic appearance of the mastic grains may have been due to a surface layer of captured granules.

In the moist state neither kind of grain gave any appearance in polarised light. But, after the slide had become dry, the mastic particles showed a shadow cross, particularly towards the edges of the grains and when the microscope was focussed slightly above their equators. The appearance vanished again on wetting the slide and seems to have been due merely to light polarised by reflexion at the edges of the spheres. Consequently the crystalline nature of the grains cannot be affirmed.

Similar experiments were made in test-tubes with aqueous solutions of from 0·12 to 0·33 % of gelatin, which were sufficiently dilute not to set for a time, the alcohol being carefully floated above the solutions. On account of the small solubility of gelatin, only small particles were obtained about 1 μ in size. The precipitate showed a tendency to form in layers, and, with the most dilute solution, a good banded precipitate of gelatin floating in water was produced by the gradual diffusion of the alcohol down the tube. The effect lasted a week, slowly disappearing as the precipitate fell to the base of the

tube. Presumably the gelatin particles were so small, or so light by adsorption of alcohol, as to remain suspended in the liquid. Since the bands settled so slowly, they could not have been produced by different rates of subsidence of the particles, and appear to have been due to the same causes as ordinary banded precipitates in gels.

Summary.

Further evidence is adduced that the reversible sol-gel transformation of the natural emulsoids is an extreme case of crystallisation and solution.

Experiments are described which demonstrate that gelatin behaves to water as does any substance to a suitable solvent. It has the definite solubility in water at room temperature (18°) of 0·12 g. per 100 cc. of solution. At 0·13 %, gelatin forms a metastable solution, with a beautiful bluish opalescence, which is a typical sol. With slight increase in concentration the metastable limit is reached and the gelatin deposited as a gelatinous precipitate, consisting of particles mostly just below microscopic size together with a few spherites reaching 1 μ. As the concentration is increased, the bulk of the precipitate grows, and the particles decrease in size, until, at about 0·7 %, the precipitate fills the solution and forms a white cloudy jelly.

Similar results were obtained with agar, and small spherites of starch by Bütschli's method.

The prediction was verified that clear gelatin gels should become opalescent and develop microscopic spherites when kept for a long time.

The experiments confirm the view that gelatin, agar, starch and silicic acid gels have a granular structure like that of a pile of shot. In other cases, the form of the ultimate particles may vary according to the crystalline habit of the gel substance.

Large granules of mastic and gamboge were grown by slow diffusion. These substances are not chemical individuals, and the grains gave no indication of crystalline structure.

An attempt to prepare smaller gelatin granules by the same method led, also, to the preparation of a banded precipitate of gelatin, which remained suspended in water for several days.

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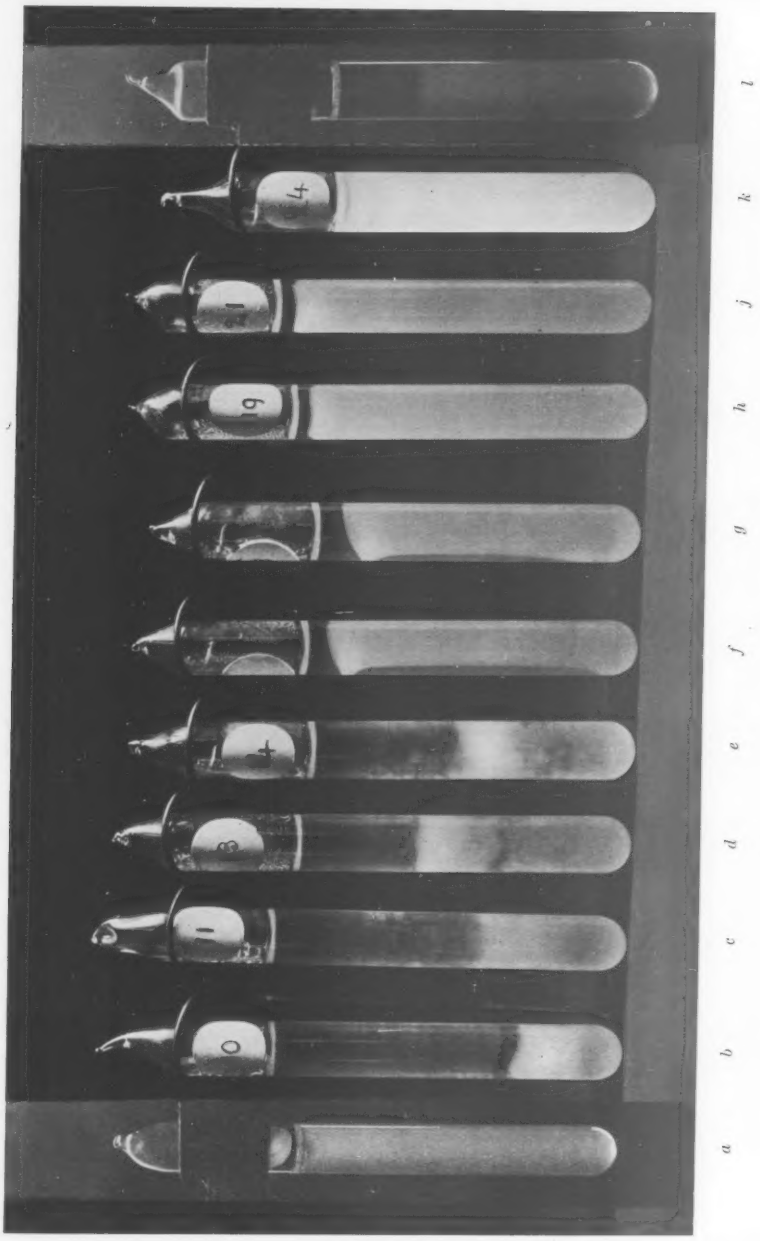
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